The Application of Biotransformations to the Synthesis of Partially Protected Sugar Derivatives.

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Man invents nothing,
he merely plagiarises nature.

Jean Battaillon

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Declaration.

The work described in this thesis is the original of the author, except where acknowledgement has been made to results and ideas previously published. The work was carried out at the Department of Chemistry, University of Warwick, between October 1st, 1987 and September 30th, 1990 and has not previously been submitted for a degree at any institution.

Summary.

This thesis describes the use of enzymes to partially deprotect or deprotect sugars. The advantages of this technique are that mild conditions may be used for the protections and that enzymatic catalysis may allow derivatives to be made which involve multi-step procedures or cannot be made using standard methods. These protected sugars can then be used to synthesise interesting derivatives. The particular aim of this project at the outset was to use biotransformations to make partially protected derivatives for the synthesis of a novel artificial sweetener, 1',4,6'-trichloro-1',4,6'-trideoxy-*galacto*-sucrose, known as sucralose.

The analysis of the products from a deacetylation reaction is a potential problem due to the number of possible products that may be formed. A new approach to this problem is discussed in Chapter Two. A combination of n.m.r. and mass spectrometry is used to analyse the products after they have first been perdeuterioacetylated by treatment with d_6-acetic anhydride. The analysis is therefore carried out on one compound, the starting material, now containing deuteriated acetate groups in place of those that were hydrolysed during the reaction.

The technique was used initially to analyse the deacetylation of sucrose octaacetate catalysed by yeast esterase. The selectivity of the enzyme for certain positions of the sugar may be determined in this way but little information can be found about the individual species that are formed. The technique can be considerably enhanced by the introduction of a chromatographic separation step. The separation of the deacetylation mixture into classes, according to the number of acetate groups, allows a much more detailed analysis of the individual components to be carried out. If the reaction shows a certain amount of selectivity then it is possible to determine the quantity of each of the individual species. This technique is used to analyse the deacetylation of glucose pentaacetate catalysed by *Aspergillus niger* lipase. The deacetylation of sucrose octaacetate catalysed by yeast esterase is also analysed in the same way.

Chapter Three describes the conversion of N-acetyl-glucosamine to N-acetyl-galactosamine. This is of interest due to the importance of this sugar in biological systems and its high cost relative to the starting material. The synthesis involves the use of an enzyme catalysed deesterification to make a partially protected intermediate, demonstrating the practical application of biotransformations in the synthesis of sugar derivatives.

Abbreviations.

n.m.r.	Nuclear magnetic resonance spectroscopy.
ppm	Parts per million.
TMS	Tetramethylsilane.
J	Coupling constant.
EI	Electron impact.
CI	Chemical ionisation.
FAB	Fast atom bombardment.
t.l.c.	Thin-layer chromatography.
h.p.l.c.	High pressure liquid chromatography.
g.l.c.	Gas-liquid chromatography.
u.v.	Ultra-violet.
$[\alpha]_D$	Specific rotation at 589nm.
c	Concentration (g/100ml).
m.p.	Melting point.
b.p.	Boiling point.
min.	Minutes.
DMF	Dimethylformamide.
DMSO	Dimethylsulphoxide.
THF	Tetrahydrofuran.
UDP	Uridine diphosphate.
NBA	p-Nitrobenzyl alcohol
Ac	Acetyl.
R	Alkyl.
Me	Methyl.
Et	Ethyl.
Bz	Benzoyl.

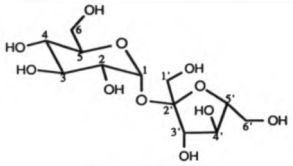
Chapter One: Introduction.

1.1: The properties of sugar derivatives.

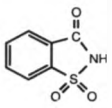
The occurrence of sugars and their derivatives in nature is extremely widespread. As one of the most important classes of compounds their various functions include uses as structural components, for energy storage and as components of cell surfaces to name but a few.[1] In addition to these natural properties they have found uses as sweeteners, surfactants, emulsifiers and tobacco improvers.[2,3,4,5] Sugar derivatives have also been found to have medicinal properties such as anti-tumour activity.[6,7,8]

The importance of glycoproteins and glycolipids as binding sites, of oligosaccharides for sorting proteins, and the effects caused by the modification of glycoproteins mean that current interest in the synthesis of sugar derivatives is high.

Of particular interest to this project has been the synthesis of artificial sweeteners. The market for non-calorific high-intensity sweeteners is very large and uses of these sweeteners include the enormous soft drinks market together with many other food applications.[9] At present there are several high intensity sweeteners on the market including saccharin, the dipeptide ester aspartame, otherwise known as Nutrasweet, and cyclamate[10] (Fig. 1.1, Table 1.1).

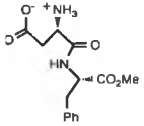


Sucrose



Saccharin

Cyclamic Acid



Aspartame

Fig. 1.1: The structures of some common sweeteners.

Aspartame	100-200
Saccharin	100-700
Cyclamate	30-80
Neohesperidin dihydrochalcone	2000

Table 1.1: The relative sweetnesses of some sweeteners. (sucrose=1)

However, these sweeteners have certain drawbacks arising from problems with toxicity and unsuitability for certain applications.[9,11] There is therefore a potential market for a novel sweetener provided that it can overcome these shortcomings. The aim of the work described in this thesis is directed at the synthesis of chloro-sugar derivatives which are improved artificial sweeteners.

1.2: Sweetness and chloro-sugars.

The chemical modification of sugars leads to compounds that may be sweet, bitter-sweet or bitter. No substantial enhancement of the sweetness of the parent sugar had been observed until in 1976 Hough and Phadnis discovered that certain halogenated derivatives of sucrose had modified sweetness.[12] The 1',4,6,6'-tetrachloro-1',4,6,6'-tetradeoxy-*galacto*-sucrose was found to be intensely sweet, with a sweetness comparable to that of saccharin, but with the advantage of not having an unpleasant aftertaste.[13] A study of a series of these derivatives revealed some intensely sweet compounds in contrast to the effect of halogenation on other sugars[14] (Table 1.2). This series of similar compounds also led to some understanding of the structural features that contribute to the sweetness of a particular compound and of the sweetness receptor site.

SUGAR	RELATIVE SWEETNESS
Sucrose	1
1'-Chloro-1'deoxy-sucrose	20
4-Chloro-4-deoxy-galacto-sucrose	5
6-Chloro-6-deoxy-sucrose	bitter
6'-Chloro-6'-deoxy-sucrose	20
4,1'-Dichloro-4,1'-dideoxy-galacto-sucrose	120
4,1',6'-Trichloro-4,1',6'-trideoxy-galacto-sucrose	650
1',4'-Dichloro-1',4'-dideoxy-galacto-sucrose	3500
1',4',6'-Trichloro-1',4',6'-trideoxy-galacto-sucrose	100
4,1',6'-Trichloro-4'-iodo-tetradeoxy-galacto-sucrose	7000

Table 1.2: Relative Sweetness of Deoxyhalo Derivatives of Sucrose.

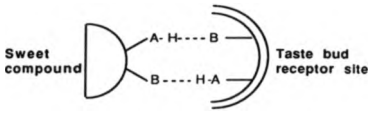


Fig. 1.2: The postulated AH, B system for sweet taste.

In 1967, Shallenberger and Acree proposed that sweetness was associated with two structural components.[15] These structural components consisted of a hydrogen bond donor, AH, and a hydrogen bond acceptor, B, separated by a distance of approximately 2Å (Fig. 1.2). These two components were postulated to initiate the sweet taste by intermolecular hydrogen bonding with a similar "glucophore" on the receptor site. In a sugar these structural components are usually

hydroxyl groups. However the role may be performed by other groups for example the halogen atoms in halogenated derivatives.

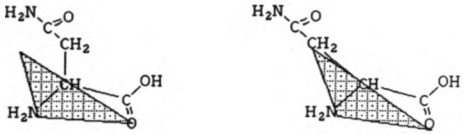


Fig. 1.3: Superpositioning of D- and L-asparagine over the same AH,B,X receptor site showing the effect of stereochemistry. In D-asparagine the structure may be superimposed on the AH,B,X glucophore. In L-asparagine the change in the conformation means that the X component is no longer in the required position.

Sweetness can however be affected by the stereochemistry of a molecule. This can be observed in the amino acid asparagine where the D-form is sweet and the L-form tasteless (Fig. 1.3). To allow for this variation, Kier proposed a third binding site, X, located 3.5Å from A and 5.5Å from B which was proposed to be hydrophobic in nature[16](Fig. 1.4).

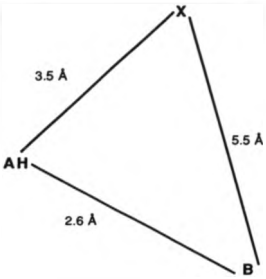
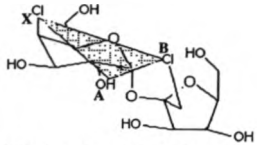


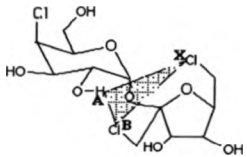
Fig. 1.4: The AH,B,X tripartite system proposed by Kier.

It was presumed that a hydrogen substituent fulfilled this role in sugars. Replacement of this substituent by a chlorine atom would be expected to increase the lipophilicity of the molecule and so facilitate the binding of the molecule to the receptor site. In sucrose the AH,B,X glucophore was proposed to be located on both the glucosyl and the fructosyl units.This was due to the relatively low sweetness of α,α-trehalose, methyl-α-D-glucopyranoside and β-D-fructofuranose which all contain either a glucose or a fructose component but not both together.[11] Selected hydroxyl groups in sucrose were then replaced by chlorine atoms using sulphonyl chloride in pyridine. It was found that the C-1', C-4 and C-6' positions were important for the enhancement of sweetness (Fig. 1.5). The combination of substitutions at these positions gave increased sweetness and the derivative with all these positions chlorinated (the C-4 position with inversion of configuration) gave the sweetest compound in the series. The 1'-chloro-

substituent can only behave as a hydrogen bond acceptor and so must be the B unit. The equatorial hydroxyl group on C-2 acts as the hydrogen bond donor, AH. The chloro- group at C-4 represents the lipophilic group X.



1',2,4-glucophore of the 1',4,6'-trichloro derivative of galactosucrose.



1',2,6',-glucophore of the 1',4,6'-trichloro derivative of sucrose.

Fig. 1.5: The two glucophores of 1',4,6'-trichloro-1',4,6'-trideoxy-*galacto*-sucrose.

The 1',4,6'-trichloro-1',4,6'-trideoxy-*galacto*-sucrose has low toxicity (the LD_{50} in mice was greater than could be administered) is non-calorific as it is not metabolised and offers the prospect of reduced dental caries. This particular derivative

has been patented under the name "sucralose" by Tate & Lyle for use as an artificial sweetener.[17,18]

All these interesting properties have increased the interest in the synthesis of sugar derivatives. There are, however, difficult problems to be overcome in the synthesis of these derivatives using classical techniques.

1.3: Selectivity in the synthesis of sugar derivatives.

Of particular relevance to this project have been the problems of selectivity in synthesis. High regioselectivity and stereoselectivity are required in the synthesis of sugar derivatives.[4] This is a difficult task to achieve owing to the presence of several functional groups which in the free sugar are generally hydroxyl groups. Unsubstituted monosaccharides contain five such hydroxyl groups. Di- and oligo- saccharides contain correspondingly more. These groups have comparatively similar reactivities which creates problems with selectivity.

To achieve the required selectivity using classical techniques involves the use of sequential protection and deprotection steps often necessitating the use of several different steps and a variety of protecting groups to provide the required level of protection. This of course can result in very long and complicated syntheses with a consequent reduction in yields.

Other problems encountered are the construction of glycosidic linkages with good selectivity with respect to the

anomeric and positional isomers and the insolubility of many sugars in the solvents commonly used in organic synthesis.

1.4: Chemical deprotection.

One method for preparing sugars with the required protection has been the deprotection of fully protected sugars by chemical methods to give the partially protected derivatives. There have been several reports of the deacetylation of peracetylated sugars to give partially deacylated products.[19]

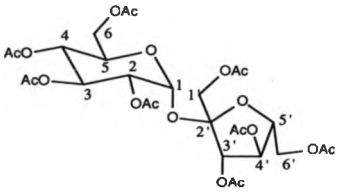


Fig. 1.6: The structure of sucrose octaacetate.

It has been reported that sucrose octaacetate (Fig. 1.6) adsorbed onto alumina from chloroform for 46 hours then eluted gave a complex mixture of products.[20] The 1',2,3,3',4,4',6-hepta-*O*-acetyl sucrose was separated from this mixture in 9% yield. Three further heptaacetates were observed, two of which were identified as 1',2,3,3',4',6,6'-hepta-*O*-acetyl sucrose (2.7%)

and 1',2,3,3',4,6,6'-hepta-*O*-acetyl sucrose (6%), indicating the hydrolysis of secondary as well as primary acetyl positions.[21]

A regio- and stereoselective deacylation of peracylated glucopyranosides at the anomeric position, leading to the 1-OH derivatives was reported by Fiancor *et. al.* using ammonia in an aprotic solvent such as acetonitrile.[22] The derivatives were formed with the α-configuration unlike previous deacylations at the anomeric position which were reported to lead to anomeric mixtures.

Further work on the deacetylation of sucrose octaacetate was carried out by Capek *et. al.* who treated a methanolic solution of sucrose octaacetate with alumina impregnated with potassium carbonate.[23] The major fraction (approximately 34%) was identified as a mixture of hexaacetates, the major component being the 1',2,3,4,6,6'-hexa-*O*-acetyl sucrose and the minor product being identified as the 2,3,4,4',6,6'-hexa-*O*-acetyl sucrose. This again shows the selective hydrolysis at a secondary position in the presence of esters of primary hydroxyl groups.

Further investigations to identify whether the hexaacetates were formed directly by deacetylation or by deacetylation of a neighbouring acetyl group followed by migration of the acetyl group were carried out by analysing the heptaacetates produced.[24] Three heptaacetates were identified, the major one being the 1',2,3,3',4,6,6'-hepta-*O*-acetyl sucrose which together with the 1',2,3,4,4',6,6'-hepta-*O*-acetyl sucrose could be further deacetylated to give the major hexaacetate product. The third heptaacetate identified was the 2,3,3',4,4',6,6'-hepta-*O*-acetyl sucrose which could be

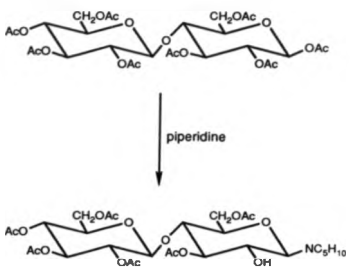
deacetylated to the minor hexaacetate product. If any other heptaacetates were formed then they were apparently rapidly deacetylated to pentaacetates or other lower acetates and so were not observed. It was also determined that there was no acetyl migration from the C-6' to the C-4' position.

Deacetylation of acetylated monosaccharides in the presence of tin oxides and alkoxides has been reported using a variety of non-hydroxylic solvents.[25] The first deacetylation occurred at the C-1 position. The next position to be hydrolysed was the C-2. This appeared to be formed directly rather than by acetyl migration from C-2 to C-1 due to the delay in its appearance. The secondary positions were found to be more reactive than the primary position although the conditions did ultimately lead to total deacetylation. It was also found that the β-anomers had higher reactivities than the α-anomers.

Another group has reported the production of the 1',2,3,4,6,6'-hexa-*O*-acetylsucrose in 20% yield by the deacetylation of sucrose octaacetate using primary amines.[26] It was again demonstrated that there was no migration between the C-4' and the C-6' positions, and that the secondary positions were more reactive with respect to hydrolysis than the primary positions. The partial deacetylation of α-D-glucose pentaacetate has been effected using cold 1M potassium hydroxide yielding 6-*O*-acetyl-α-D-glucose.[27]

Cellobiose octaacetate may be deacylated by reaction with piperidine (Scheme 1.1), as may glucose pentaacetate.[28] In both cases the product formed is deacetylated at the C-2 position and piperidine is substituted in at the C-1 position. There is

evidence that the deacetylation occurs before the piperidine substitution.



Scheme 1.1: The reaction of cellobiose octaacetate with piperidine.

However, these methods of deacetylation are restricted both in the position of hydrolysis and the selectivity, leading to poor yields of individual partially deprotected derivatives. This is particularly true of deprotection in the glucosyl portion of sucrose.

1.5: Acyl migration.

There are many examples of intramolecular migrations of substituent groups in carbohydrate molecules.[29,30] Many of these involve the migration of acyl groups. These are obviously a potential nuisance as migration may mean that any selectivity in a reaction is lost through isomerisation of the product.

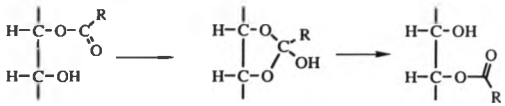


Fig. 1.7: Acyl migration *via* an ortho-acid intermediate.

Acyl migrations appear to have been originally discovered by Fischer, who proposed that the mechanism in diols proceeded *via* an orthoacid intermediate.[31](Fig. 1.7) The intramolecular nature of the migration was established by a radioactive labelling study.[32] The acyl migrations may be either acid or base catalysed and the conditions of many deacylations provide suitable conditions for migrations to occur.[33]

The rate of the acyl migration depends on several factors such as the solvent, the pH of the medium and the proximity and configuration of the two centres involved in the migration.[34,35,36,37] The products are often unpredictable owing to the occurrence of successive migrations.

Acyl migrations often involve the migration of a group from a secondary to a primary position. Acyl groups attached to any of the oxygen atoms in a D-glucose ring may migrate. The acyl migrations in the glucose ring are most often observed in the direction away from C-1 towards C-6, although in theory the process is reversible. Examples of migration in the reverse direction have been observed such as the migration from C-6 to C-4.

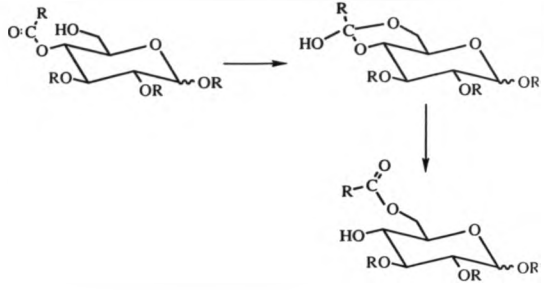


Fig. 1.8: The C-4 to C-6 acyl migration.

The most stereochemically favourable migrations are those from O-4 to O-6 (Fig. 1.8) and from O-1 in the α-configuration to O-2 and these have been well established.[29,30] Migration between vicinal diols occurs more readily when the groups are *cis* rather than *trans* to each other. This is probably because there is less strain in the *ortho*-acid intermediate for *cis* migrations. Migration of an acyl group from nitrogen has also been observed, as has the reverse process[37,38](Fig. 1.9).

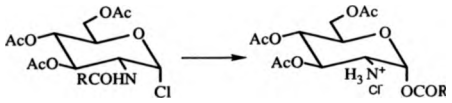


Fig. 1.9: Migration of an acyl group from nitrogen.

Work by Tsuda and Yoshimoto also confirmed the *trans* migration from O-2 to O-3 although there was no observation of any migration between O-3 and O-4 or from β-O-1 to O-2.[36] Migration from O-3 to O-6 was observed in *O*-myristoyl-D-glucose and glucosides. This migration apparently did not proceed *via* the O-4 intermediate as no O-4 acylated intermediates could be detected. There was also evidence for the reverse process (O-6 to O-3 migration) to a small extent. It was found that the *O*-myristoyl-β-glucose was stable to acyl migration. On heating in pyridine the myristoyl group was observed to migrate first to O-3 and then to O-6, although there was no evidence for a direct migration from β-O-1 to O-6.

In a study of various derivatives of sucrose at Tate & Lyle several migrations were observed.[39] There were large differences in the rates of these migrations. The rates of these migrations were found to be:-

O-4 to O-6 > O-3 to O-4 , O-2 to O-3 > O-3' to O-1'.

It was also found that by controlling the conditions under which a reaction was performed the migrations could be prevented. For example, if the pH of an aqueous solution of sugar esters was kept below 5.0 or above 8.0, migrations were greatly reduced. Obviously products must also be removed from solution as soon as is possible, to prevent migrations when the conditions are such that they may occur.

1.6: Enzymatic deacylations.

An alternative to chemical deacylation is to employ biotransformations, which may be defined as "...selective enzymatic conversion of natural or chemically synthesised substrates into defined products on a preparative scale using whole cells or isolated enzyme systems".[40,41] There are many different known enzymes and in 1955 the Enzyme Commission conveniently gave each enzyme a unique E.C. number (E.C. w.x.y.z.). All enzymes belong to one of six classes (w=1 to 6) dependent on the type of reaction catalysed:[42]

(1) Oxidoreductases
(2) Transferases
(3) Hydrolases
(4) Lyases (elimination)
(5) Isomerases
(6) Ligases (also known as synthetases)

One of these classes, the hydrolases, consists of enzymes which have the potential to catalyse the hydrolysis of ester linkages. This class includes enzymes such as esterases, lipases and proteases. Esterases catalyse the hydrolysis of glycerol esters. However, they are also capable of hydrolysing a wide range of other substrates. Lipases are a special class of esterase which in nature act on glycerides at a membrane surface. Proteases naturally catalyse the formation of peptide bonds. However, they are also capable of hydrolysing esters. The hydrolysis of sugar esters may be the natural activity for some

enzymes but others, designed to catalyse other reactions, will also be able to catalyse these de-esterifications.

The advantages of using enzymes arise from their selectivity together with their ability to function under mild conditions.[43,44] The regio- and stereoselectivity which can be achieved with an enzyme can be completely different from those observed using any chemical methods.[45]

Enzymes can therefore be used to deacylate protected hydroxyl groups in different molecules and there have been several reports of the use of enzymes to catalyse the deacylation of sugar derivatives including both mono- and disaccharides. Frohwein *et. al.* found that several enzymes were capable of catalysing the deacetylation of various mono- and disaccharides.[27,46] Reese *et. al.* also found that enzyme systems from certain microorganisms were able to catalyse the deacetylation of cellobiose acetates.[47]

In 1968 Fink and Hay used wheat germ lipase to catalyse the deacetylation of esterified mono- and disaccharides.[48,49] Wheat germ lipase had been found to hydrolyse glyceryl and aliphatic esters and Mounter and Mounter had concluded that wheat germ lipase contained a single enzyme activity.[50] For the study of deacylations of peracylated disaccharides the α- and β-glucosidase activities were removed using preparative polyacrylamide gel electrophoresis. Fink and Hay confirmed that wheat germ lipase exhibited maximum activity towards acetyl esters. They also found by studying homogeneous and heterogeneous substrate systems that the physical state of the substrate did not affect the rate of the enzyme-catalysed reaction. This was in agreement with the results of Mounter and

Mounter.[50] Solvents such as acetone or acetonitrile were used to increase dissolution of the substrate at high concentrations, although in general the solubilising agent was not essential.

As expected from the chemical reactivity, they discovered that the anomeric acyl group was most easily hydrolysed by the enzyme. For the other positions the relative rate of deacylation, determined from the identification of the partially acylated products was, for glucose pentaacetate :-

C-1 > C-6 > C-4 > C-3 > C-2

and for peracetylated maltose and cellobiose :-

C-1 > C-6', C-6, C-4' > C-3', C-3 > C-2', C-2.

However, they had difficulty in determining the difference in reactivity between similar positions. The deacylation at C-4 in glucose pentaacetate hydrolysis proceeded faster than the chemical deacetylation and this was thought to be because the C-4 acetyl group was favourably positioned for hydrolysis. They were unable to rule out the possibility that acyl migrations occurred during the reaction. However, they did consider that the structures of the products suggested that migrations did not occur to any large extent.

It would however seem likely, considering the ease of the C-4 to C-6 migration, that the reactivity of the C-4 position was effectively enhanced by this migration process. Although no 3,4,6-tri-*O*-acetyl derivative was identified, this does not provide sufficient evidence for there being no migration.

The substrate size was also found to be an important factor in the catalytic process. The binding constant from Michaelis-Menten kinetics (K_M) was found to be much smaller for disaccharides. Substrates were also able to change their

binding to the enzyme during a deacetylation reaction. This may then affect the reactivities of the remaining acetyl groups.

Most of the more recent work on the selective enzymatic deacylation has been carried out on monosaccharides. Sweers and Wong studied the hydrolysis of methyl-2,3,4,6-tetra-*O*-pentanoyl-D-hexosamines using a lipase from *Candida cylindracea*.[51] The reactions were performed at room temperature in a phosphate buffer. Regioselective deacylation of the sugar to produce the 6-OH derivative was achieved in yields of 80-90%. They found that the best substrates for the enzyme were the octanoyl derivatives but that it was more convenient to work with the pentanoyl derivatives. They considered that this method provided a better means of synthesizing partially protected hexopyranoses than those previously described. Having observed this regioselectivity in the hydrolytic reaction, the reverse procedure, esterification, was attempted to see if the regioselectivity was maintained. This approach will be discussed in a later section.

Shaw and Klibanov used glucose pentaacetate (Fig.1.10) as the substrate for enzymatic hydrolysis, and were able to produce glucose di-, tri- and tetraacetates on a gram scale.[52] They used four enzymes to catalyse the reactions :- lipases from *Aspergillus niger*, and *Rhizopus oryzae*, wheat germ lipase and porcine liver carboxyl esterase. These reactions were performed at 30°C in phosphate buffer at pH 7.9. Significant accumulations of various acetate classes were found at different degrees of conversion, and with the different enzymes.

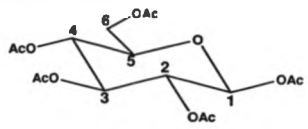


Fig. 1.10: The stucture of β-D-glucose pentaacetate.

Larger scale experiments were carried out to prepare individual glucose esters. The lipase from *A. niger* was used because of its relatively low cost and significant accumulations of the tetra-, tri-, and diacetates during the deacylation reaction.

Analysis of the products carried out by gas chromatography and ^{13}C n.m.r. spectroscopy identified the major tetraacetate from the hydrolysis of glucose pentaacetate as 2,3,4,6-tetra-*O*-acetyl glucose. The anomeric acetoxy group was, as expected, hydrolysed first. The triacetate fraction was found to be a mixture of 2,4,6- and 3,4,6-tri-*O*-acetyl glucose. The diacetate was shown to be the 4,6-diacetate. At 20% conversion 74% of the mixture was composed of tetraacetate, at 40% conversion 60% of the mixture was triacetate and at 60% conversion 30% was diacetate.

Shaw and Liaw found that the yield of the 2,3,4,6-tetra-*O*-acetyl glucose could be greatly improved by performing the lipase catalysed reaction in an organic solvent.[8] Using ethyl acetate/0.1 M potassium chloride solution (7:3) as the solvent for the reaction, the tetraacetate could be produced in 100% yield. Different alcohols were used to investigate the selectivity of the enzyme. The identification of the product as the 2,3,4,6-

tetraacetate was confirmed by the anomerisation of the product indicating that the acetoxy group at C-1 had been hydrolysed.

Csuk and Glänzer reported the deacetylation of α-acetoxy-furanurono-6,3-lactones using lipases and lyophilised yeast.[53] Lipase AP6 from *Aspergillus* sp. caused 94% deacetylation of the substrate in 3 hours, and the lipase P from *Pseudomonas* sp. catalysed 84% deacetylation after 30 hours.

Tomic *et al.* used rabbit serum esterase to hydrolyse 2,6-di-pivaloyl-α-D-glucopyranose selectively to give the 2-pivaloyl and the 6-pivaloyl derivatives in a ratio of 6:1.[54] This ratio remained the same regardless of the reaction time or reaction concentration. Hennen *et al.* reported the selective deacetylation of the primary acetyl groups of peracetylated methyl furanosides.[55] The lipase from *C. cylindracea* was used to catalyse the reaction in a 10% DMF solution. In the case of the 2-deoxyriboside series the 3-*O*-acetyl group was selectively hydrolysed.

The use of DMF was found to enhance the regioselectivity of the deacylation of pyranoses. Glucose pentaacetate was exclusively hydrolysed at C-1, using porcine pancreatic lipase in 10% DMF, to give the 2,3,4,6-tetraacetate in 70% yield. Similar selectivity was found with six other peracetylated hexoses. The same hydrolysis of the 1-acetoxy group was observed with tetra-*O*-acetyl-D-xylofuranose and tetra-*O*-acetyl-D-ribofuranose using *A. niger* lipase. An interesting result was that the *C. cylindracea* lipase hydrolysed both the 4-*O*- and the 6-*O*-acetyl groups from α-D-glucose pentaacetate producing the triacetate in 73% yield. This result may again be due to the C-4 to C-6 acetyl migration followed by deacetylation.

An investigation of the influence of the size and position of the acyl group using lipases from *C. cylindracea* and porcine pancreas was carried out by Kloosterman *et. al.*[56] Porcine pancreatic lipase was found to hydrolyse primary *n*-butyryl esters but not acetyl esters at C-6 in 1,2-3,4-diisopropylidene-α-D-glucopyranose (Fig. 1.11). When 1,2-5,6-diisopropylidene-α-D-glucofuranose was treated with *C. cylindracea* lipase the secondary butyryl ester was hydrolysed but the corresponding acetyl ester was not. Porcine pancreatic lipase hydrolysed both esters. The use of organic solvents such as hexane, acetone or dichloromethane led to slower rates and lower yields.

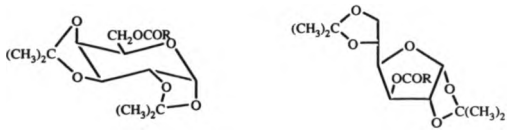


Fig. 1.11: 1,2-3,4-diisopropylidene-α-D-glucopyranose and 1,2-5,6-diisopropylidene-α-D-glucofuranose.

Using *C. cylindracea* lipase it was possible to hydrolyse, selectively, a primary butyrate group in the presence of a secondary acetate. Apparently there was some migration of the acetyl group from C-3 to C-5 under the reaction conditions. The influence of the size of the acyl group on the enzymatic hydrolysis was shown by the selective deacylation of a secondary butyryl group in the presence of a primary pivaloyl group.

secondary butyryl group in the presence of a primary pivaloyl group.

Interest in chloro-deoxy-sugars, in particular 1',4,6'-trichloro-1',4,6'-trideoxy-*galacto*-sucrose-"sucralose"-mentioned earlier, led Tate & Lyle to investigate the possibility of synthesizing partially protected sucrose derivatives from sucrose octaacetate.[39] Using sucrose octaacetate (Fig. 1.12) as a suspension in water, or water in combination with an organic solvent it was found that selective deacylation of five of the eight acyl groups was possible. Different deacylation products were found using different enzymes. Acetates at the C-2, C-3 and C-3' positions did not appear to be hydrolysed by any of the enzymes investigated.

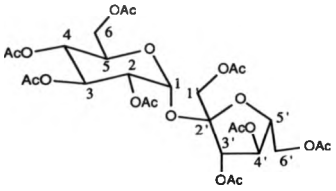


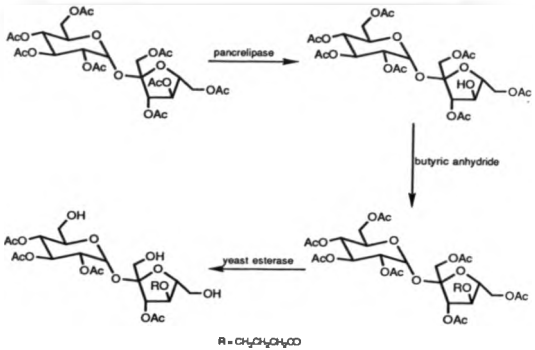
Fig. 1.12: Sucrose octaacetate.

Using subtilisin Carlsberg to catalyse the deacetylation, the C-1' position was preferentially hydrolysed. This was followed by hydrolysis of the 6'-acetate. The 4-acetate was hydrolysed to a small extent using a different strain of subtilisin Carlsberg.

A yeast esterase obtained from Glaxo specifically catalysed the deacetylation of the 1'-, 4'-, 6- and 6'-acetoxy groups to produce 2,3,3',4-tetra-*O*-acetyl sucrose. Some hydrolysis of the C-4 acetate was observed but this was probably due to migration of the group from C-4 to C-6 subsequently followed by hydrolysis. This particular tetraacetate has been identified as a natural product.[57,58]

Wheat germ lipase was found to hydrolyse the 4'-, 6'- and 1'-acyl groups from the furanose ring. The analysis of this particular hydrolysis will be discussed in greater detail in Chapter 2.

Of the other enzymes studied pancrelipase (Scientific Protein Laboratories) selectively cleaved the 4'-acetyl group and *Aspergillus meleus* protease A catalysed the production of a large number of products, the major one being identified as 1',2,3,3',4'-pentaacetate.[39] The use of *Aspergillus usami* lipase as the catalyst led to the same pentaacetate being formed together with the 2,3,3',4',6'-pentaacetate. *Mortierella vinacea* melibiase produced 1',2,3,3',6-penta-*O*-acetyl sucrose in addition to the 1',2,3,3',4'- and the 2,3,3',4',6'- pentaacetates. Amylase catalysed the production of 1',2,3,3',4,6-hexaacetate and α-galactosidase hydrolysed sucrose octaacetate to give two major products. These were identified as the 1',2,3,3',6'-pentaacetate and the 1',2,3,3',4'-pentaacetate. The enzyme rapidase, a crude fungal alpha amylase preparation (Miles Laboratories), to catalyse the deacetylation led to the 1',2,3,3',4',6-hexaacetate being isolated in 5% yield. These deesterifications did not, however, produce the partially protected intermediates required for the synthesis of sucralose.



Scheme 1.2: The synthesis of 2,3,3',4-tetra-*O*-acetyl,4'-*O*-butyryl sucrose, an intermediate in the synthesis of sucralose, using two enzymes.

The use of two or more enzymes, either sequentially or simultaneously, is also a possible means of producing the required product, as is the combination of an enzymatic and a chemical deacetylation process. Two different enzymes have been used in successive manner by Tate & Lyle to synthesize one of the intermediates in the synthesis of sucralose. The selectivity of certain enzymes for certain acyl groups was also utilised (Scheme 1.2). Sucrose octaacetate was treated with pancrelipase to produce the 4'-OH derivative. This was then treated with butyric anhydride to reprotect the C-4' position. This esterified compound was then hydrolysed with the yeast esterase. The product was the 2,3,3',4-tetra-*O*-acetyl,4'-*O*-

butyryl sucrose. The yeast esterase did not catalyse the hydrolysis of the butyrate group.

The regioselective deacetylation of sucrose octaacetate has also been carried out by Kloosterman *et. al.*[5] A screen of commercially available lipases and esterases was carried out in aqueous buffer. The reactions were monitored by t.l.c. over a period of five days. Most of the enzymes screened did not hydrolyse sucrose octaacetate to an observable extent. Two enzymes, pig liver esterase (Sigma) and another esterase (E122 from Novo) hydrolysed the substrate but produced a mixture of products with no obvious selection. *C. cylindracea* lipase (A6, Amano) hydrolysed the substrate completely to sucrose.

Regioselective deacetylation was achieved using wheat germ lipase (L-3001, Sigma) which hydrolysed sucrose octaacetate to give one major product in about 45% yield. The major product isolated in 37% yield was identified as 2,3,3',4,6-penta-*O*-acetyl sucrose. A minor product 1',2,3,3',4,6-hexa-*O*-acetyl sucrose was also identified. The addition of calcium or iron ions did not have any effect on the enzyme activity or selectivity. The addition of lipophilic cosolvents had no marked effect on the enzymatic activity or selectivity. This is in contrast to the effect on the deacylation of glucopyranosides catalysed by *C. cylindracea* lipase previously reported.[56] This confirmed an earlier report that wheat germ lipase is an esterase.[50] Immobilisation on agarose caused the rate of conversion of sucrose octaacetate to be very slow.

C. cylindracea lipase (lipase AY30, Amano) was also found to produce a major product from the hydrolysis of sucrose octaacetate. The product was identified as 1',2,3,3',4,6,6'-hepta-

O-acetyl sucrose. It was recovered in a yield of 35%. This is another example of a secondary ester being preferentially hydrolysed in the presence of primary esters.

Examples can be found where sucrose octaacetate has been deacylated at both primary and secondary positions in either the pyranose or the furanose ring. It is apparent from many of these results that one of the secondary acyl groups in sucrose octaacetate, the C-4' one, has a reactivity comparable to and often greater than those at the primary positions.

Different enzymes are seen to have different selectivities and the duration of the reaction and other factors such as co-solvents and the acyl group used can affect the products produced from a reaction. These results show the potential of enzymatic deacylations as a means of synthesizing sugar derivatives.

1.7: Enzymatic acylations.

A further approach to the synthesis of partially protected sugar derivatives is by the regioselective acylation of a sugar.[59] In order to esterify a hydroxyl group enzymatically several major factors must be taken in to account. The enzyme is in effect being made to work in reverse and to make the acylation possible it also has to work in a non-aqueous environment. Water is involved in the equilibrium of the ester/alcohol conversion so that if there is any water present in the system in large amounts the hydrolysis of the ester will become the energetically favourable reaction.[60] This also applies to the processes of aminolysis, thiotransesterification and oximolysis.

However, it is necessary to have some water present in the system to maintain the catalytic activity of the enzyme. This water forms a protective shell around the enzyme that prevents it from being denatured. Only a very small amount of water is required and this will already be present in all but the driest solvent.

In 1966, Dastoli and Price observed enzymatic activity of chymotrypsin and xanthine oxidase in anhydrous non-polar organic solvents.[61,62] There have now been many examples of enzymes functioning in low water systems to catalyse a variety of different reactions. Many enzymes are capable of working in organic solvents containing little or no water. In some circumstances the enzymes actually acquire enhanced stability, altered substrate and enantiomeric specificities and the ability to catalyse unusual reactions.[63,64] Klibanov also reports the property of molecular memory whereby an enzyme is "imprinted" by a ligand prior to lyophilisation.[65] When placed in an organic solvent the enzyme remains in the same conformation as in the presence of the ligand in aqueous solution.[66] This apparently leads to greatly enhanced reactivity.

Klibanov has suggested a series of rules for the synthetic use of enzymes in organic solvents.[44] These are that the solvent is carefully chosen; a hydrophobic solvent is preferable because hydrophilic solvents "strip" water molecules from around an enzyme thereby reducing its activity. The effect of hydrophilic solvents is different for each enzyme and may be overcome by adding a small amount of water to the solvent.

A second rule is that an enzyme will function best if it is recovered from an aqueous solution which has a pH that is

optimal for the enzyme activity. This is best done by lyophilisation. This "pH memory effect" is supposedly due to the enzyme acquiring the ionisation state corresponding to the optimal pH and retaining this state after lyophilisation and exposure to the organic solvent.

The third rule is that the reaction mixture must be agitated so that a fine dispersion of the enzyme may be achieved. This is so that the diffusion of the substrate to the enzyme is not too limited by the enzyme being suspended in an organic solvent rather than being dissolved in water. It is claimed that the enzymatic transesterifications follow Michaelis-Menten kinetics closely.[65] This indicates that the reaction proceeds *via* an enzyme substrate complex (Fig. 1.13).

$$E + S \underset{}{\overset{K_M}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

Fig. 1.13: The Michaelis-Menten mechanism.

The thermostability of enzymes in organic solvents is often dramatically increased. For example, chymotrypsin has a half-life of several hours in anhydrous octane at 100°C as compared to minutes in water at 60°C. Lipases may also remain catalytically active at temperatures up to 100°C in organic solvents. This is because the conformational mobility required to denature the enzyme by irreversible unfolding is highly restricted in anhydrous organic solvents.[65]

This ability of enzymes to catalyse reactions even in anhydrous organic solvents dramatically increases the range of

reactions which may be carried out using enzymes as catalysts. The enzymatic acylation of hydroxyl groups of simple alcohols with both stereo- and regioselectivity is well reported in the literature.[67,68] It has now been applied to the more complex task of selectively acylating sugars.

Therisod and Klibanov were able to selectively acylate the primary hydroxyl groups of various unprotected monosaccharides.[69] The reactions were catalysed by dried porcine pancreatic lipase in anhydrous pyridine at 45°C. The acyl donating compound for the transesterification was 2,2,2-trichloroethylbutyrate. Yields of 50-90% were achieved on a gram scale. The acylation rate could be increased by increasing the temperature, the lipase concentration, the substrate concentration or the acyl donor concentration. Fructose was found to be acylated at both the primary positions.

The same workers then carried out a study of the acylation of secondary hydroxyl groups of sugars.[70] For this study they used sugars which were "blocked" in the primary C-6 position. The derivatives synthesised by the above enzymatic transesterification were suitably protected. A preference was shown for acetone, tetrahydrofuran or methylene chloride as the solvent over pyridine. It was expected and found that the lipases would be more reactive in these solvents.

Four enzymes were able to catalyse the acylation of 6-*O*-butyryl glucose at the secondary hydroxyl positions. Using *Chromobacterium viscosum* lipase 6-*O*-butyryl glucose was exclusively acylated at the C-3 hydroxyl to give 3,6-di-*O*-butyrylglucose. *A. niger* lipase produced very similar activity. Porcine pancreatic lipase however catalysed acylation to give

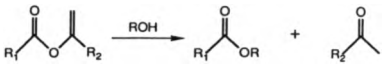
90% of the 2,6-di-*O*-butyryl derivative. *C. cylindracea* lipase was slightly more reactive towards the C-2 position but there was little selectivity shown. If a bulkier group such as a tertbutyldiphenylsilyl- group was used to block the C-6 position then *C. cylindracea* lipase acylated the C-2 position almost exclusively.

The acylation of mannose and galactose derivatives in an identical manner showed some selectivity, but not as much as was shown for the glucose derivatives. It was also found that it was possible to hydrolyse, selectively, the 6-*O*-butyryl group from the above diesters using the method of Sweers and Wong (see earlier).[50] This leaves a monoester protected by an acyl group at one of the secondary positions. The C-3 position of 6-*O*-trityl glucose could also be selectively acylated using *C. viscosum* lipase. Detritylation leads to 3-*O*-butyryl glucose again having a secondary position protected.

When both primary and secondary positions were available for acylation all the enzymes showed a preference for the primary position. However whereas *C. cylindracea* lipase would not undergo further acylation to the diester the other three lipases formed a mixture of mono- and diesters. The regioselectivity for the secondary hydroxyl groups was similar to that found before.

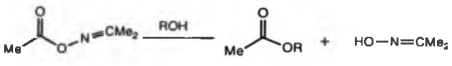
Wong *et. al.* were also able to acylate sugars at the primary position with 79% regioselectivity.[63] Their solvent of choice was pyridine to overcome the problem of sugar solubility and to maintain enzyme activity. The activity was found to be enhanced by the addition of benzene. Vinyl acetate was used as the acyl donor with *C. cylindracea* lipase as the biocatalyst.

Vinyl esters were found to have a rate of transesterification up to 100 times greater than ethyl esters and five times faster than isopropenyl esters.[71] The additional advantage of using acetyl donors such as vinyl acetate, isopropenyl acetate and oxime acetates is that the *trans*-esterification is irreversible (Scheme 1.3). This is because the donor groups are not alcohols so transesterification of the product does not occur and as the conditions are essentially anhydrous, hydrolysis is not possible.



isopropenyl acetate: $R_1=R_2=CH_3$

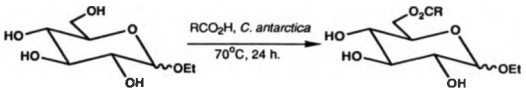
vinyl acetate: $R_1=CH_3$, $R_2=H$



Scheme 1.3: The irreversibility of the acylation process using isopropenyl acetate, vinyl acetate or oxime acetates.

Björkling *et. al.* have also reported the regioselective acylation of alkyl glucosides at the primary C-6 position to yield monoesters in 90% yield.[72,73](Scheme 1.4) Their process was solvent free with the substrate and lipase being mixed together with the long chain (C-8 to C-18) fatty acid used for the acylation. The lipase used was from a strain of *Candida antarctica*, which was immobilised on a macroporous acrylic or

phenolic resin. This enabled the enzyme to be used several times with no noticeable loss of activity. Ethyl-D-glucopyranoside was used as substrate and was found to be very much more reactive than methyl-D-glucopyranoside, or glucose itself, possibly owing to the increased solubility. When either *n*-propyl- or *n*-butyl-D-glucoside were used a significant amount of diester formation was observed. This was identified as the 2,6-di-*O*-acyl-derivative. The yields of the monoesters increased with the increasing chain length of the fatty acid. A possible reason for this was thought to be that the increased solubility of the shorter chain fatty acids in water affected the pH of the water bound to the enzyme and maybe dissolved the water at the enzyme surface causing a decrease in the activity.



R = n-C_nH_{2n+1} n=7 to 17

Scheme 1.4: The specific acylation of ethyl-D-glucopyranoside using long chain fatty acids.

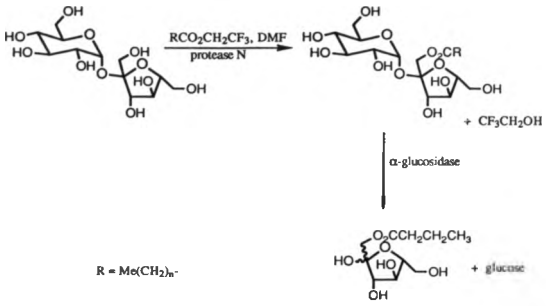
Four other enzymes catalysed the reaction but either they were less specific producing more diester or they gave very low conversions compared to *C. antarctica* lipase. The reactions were carried out at 70°C under reduced pressure. Water generated from the reaction was removed *in vacuo*.

Carrea *et al.* used subtilisin, a protease from *Bacillus subtilis*, to catalyse the enzymatic synthesis of 1'-*O*-acyl sucrose.[74] This position has been considered to be the least reactive of the three primary positions of sucrose from the reaction of sucrose in chemical reactions, for example, tritylation, silylation, acetylation with acetic anhydride or chlorination with sulphonyl chloride.

The enzyme was able to accept various different trichloro- and trifluoroethyl esters as the acyl donor. The best results were obtained using trifluoroethyl butyrate with the yield of the 1'-monoester decreasing with the increasing size of the acyl group. The regioselectivity and rate were also affected by increasing the length of the aliphatic chain of the acyl group. The reactions were carried out in dry dimethylformamide at 45°C (Scheme 1.5). The enzyme used, protease N - a crude form of subtilisin - was found to be inactivated after three days in pure solvent but under reaction conditions inactivation occurred much faster.

Chromobacterium viscosum lipase was found to catalyse the regioselective acylation of sucrose to give 1'-*O*-hexanoyl sucrose in 31% yield using acetone as solvent. A longer chain acyl donor trifluoroethyl laurate was also used for the transesterification.

The 1'-*O*-sucrose esters were substrates for yeast α-glucosidase and this enzyme was used to catalyse the synthesis of 1-*O*-acyl fructose compounds (Scheme 1.5). The direct enzymatic acylation of fructose with trichloroethyl butyrate produced a mixture of two compounds: the 1-*O*-butyryl fructose and 6-*O*-butyryl fructose in a ratio of 8:2.



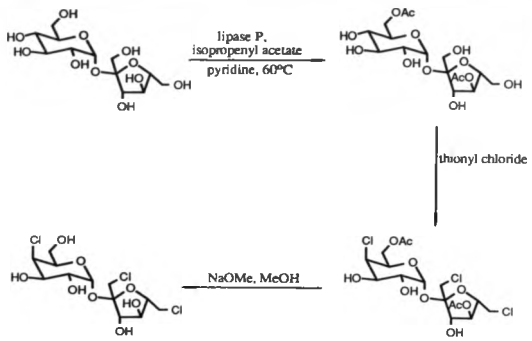
Scheme 1.5: The selective 1'-*O*-acylation of sucrose as a means of synthesising 1'-*O*-fructose.

The regioselective enzymatic acylation of octyl-α-D-glucopyranoside has recently been reported.[2] Five lipases were tested for the acylation. Porcine pancreatic lipase was found to prefer medium length straight chain esters, *Pseudomonas* lipase preferred esters with an additional carbonyl group such as a succinate ester. *Mucor miehei* lipase seemed to have no preference. *C. cylindracea* and *A. niger* lipases were found to have very low activity which only increased on the addition of water to the reaction.

The best results were achieved using porcine pancreatic lipase in THF with ethyl octanoate, and for succinate and acetoacetate esters using *M. miehei* lipase, with 1,2-

dichloroethane and without cosolvent respectively. The acetoacetate derivative was found to be a mixture of the 2-*O*- and 6-*O*-acyl derivatives. If the acylations were continued for longer than three days, considerable diesterification was observed. The β-anomer of 1-*O*-octanoyl-D-glucopyranoside was acylated more slowly and with more by-product formation. These compounds were investigated for their potential surface activity and liquid crystal properties. Tate & Lyle have also investigated the use of selective enzymatic acylations as a means of synthesising the intermediates for the synthesis of sucralose.[75]

Selective chlorination of sucrose at certain positions without protecting the other positions has been found to be possible.[76] For example the hydroxyl groups at the C-1', C-4 and C-6' positions may be selectively chlorinated in the presence of unprotected hydroxyl groups at C-2, C-3 and C-3'. This would produce the trichloro sucrose derivative, Sucralose. Unfortunately the hydroxyl group at C-6 must be protected to prevent chlorination at this position and some traces of chlorination have been found at C-4' so this position really needs to be protected as well.



Scheme 1.6: The synthesis of sucralose using a selectively acetylated derivative.

A selective acylation process to protect the C-4' and C-6 positions would therefore allow the synthesis of a sucralose derivative.[75] Using a bacterial lipase derived from *Pseudomonas* sp. (lipase P, Amano) acylation initially at the C-6 position followed by acylation at C-4' has been achieved to give the 4',6-diacetate. The acylation was carried out in pyridine at 60°C using either isopropenyl acetate or trichloroethyl esters as the acyl donors (Scheme 1.6). After four days a yield of 8% of 4',6-di-*O*-acetyl sucrose and 33% of the monoester, 6-*O*-acetyl sucrose were recovered. The monoester could be converted to the 4',6-diester in 47% recovered yield by a similar acylation reaction at 60° C for six days followed by the addition of more enzyme and another 24 hours of reaction. A tetraacetate,

2,3,3',6-tetra-*O*-acetyl sucrose may also be acetylated at the 4'-hydroxyl group to give 2,3,3',4',6-penta-*O*-acetyl sucrose in 55% yield. This again is one of the key intermediates in one synthetic route to sucralose.

1.8: Analysis.

A major problem with both acylation and deacylation reactions is the identification of the products which are formed, in particular when several products are made. This is, of course, the case more often than not with these types of reaction.

In a monosaccharide such as glucose there are five different positions which can be acylated or deacylated. In theory there could be as many as 32 (2^5) different products in the reaction mixture from the deacetylation of glucose pentaacetate. For a disaccharide such as sucrose there could be as many as 256 (2^8) different products including the substrate from the deacetylation of the peracetate.

Due to the similar nature of many of the hydroxyl groups within a sugar many of these different compounds, particularly those with identical numbers of hydroxyl groups free, will have very similar properties.[77] This obviously can lead to difficulties with separation using chromatographic techniques and with the analytical techniques normally used. The different techniques used for the separation and purification of sugar derivatives include gas-liquid chromatography (g.l.c.), high performance liquid chromatography (h.p.l.c.), column and thin-layer chromatography (t.l.c.).

Thiem *et. al.* were able to separate a group of peracetylated monosaccharides using h.p.l.c. on silica gel columns.[78] The compounds were detected by ultra-violet detection at 223nm. There is a weak chromophore from the acetate groups at this wavelength. The problem with operating at this wavelength is that the chromophore is so weak that it can be lost if any highly u.v. absorbing compounds are present.

Analysis of the products from acylation reactions were carried out by Adelhorst *et. al.* using an amino-silica (SiO_2/NH_2) h.p.l.c. column and a refractive index detector.[73] De Nijs *et. al.* used a C18 silica column also with refractive index detector for their analyses of acylation mixtures.[3]

Gas/liquid chromatographic separations have been carried out using various types of column packing. Analysis using this technique requires that the samples are first derivatised, usually using a silylating agent such as 1,1,1,3,3,3-hexamethyldisilazane. The sugar derivatives are then sufficiently volatile for them to be carried in the gas phase through the column.

The problem with these techniques is that it is extremely hard to separate all the different products within a mixture and to be certain that no two peaks are overlapping or that a major peak is not hiding a minor peak. Complete separation of all the products in a mixture, ranging from the peracetate to the free sugar, is likely to be an exceptionally complex task owing to the large difference in polarity between the two extremes.

The actual analysis of individual sugars can also be a complex problem as the ^1H n.m.r. spectra often contains several overlapping signals, making the identification harder. In 1969

Binkley *et. al.* recorded the ^1H n.m.r. spectrum of sucrose octaacetate in d_6-benzene, d-chloroform and d_6-acetone at 100MHz and 220MHz.[79] The signals due to H1, H2, H3, H4 in the α-D-glucopyranosyl fragment and due to H3 and H4 in the β-D-fructofuranosyl fragment were individually assigned. The spectra of acetylated derivatives of sugars were observed to have signals in three different regions:- the acetyl protons at about 2.0ppm, the methylene protons and those protons attached to carbon atoms on which the hydroxyl group is not acetylated, i.e. hydroxyls or ethers, at about 4ppm, and the region due to methine protons with the hydroxyl group acylated together with the anomeric protons from 4.5 to 6.5 ppm. A variation was seen in the spectrum of sucrose octaacetate when it was recorded in different deuteriated solvents. Using d_6-benzene instead of d-chloroform better resolution of the methine proton signals was achieved. This has also been observed by Sanders who fully assigned the proton signals.[80]

Suami *et. al.* established a means to identify the acetyl proton signals by using specifically deuteriated acetyl sucrose derivatives.[81] This approach had been previously used to identify the signals for glucosamine derivatives.[82] This technique uses the fact that deuteriated acetate will give no signal in the ^1H n.m.r. spectrum. The ^1H n.m.r. spectrum of sucrose octaacetate showed only six signals in the acetate region because some of the signals were not resolved. A known sucrose pentaacetate, which was treated with d_6-acetic anhydride in pyridine to give penta-*O*-acetyl-tri-*O*-deuterioacetyl sucrose, contained four peaks in the acetyl region of the ^1H n.m.r.

spectrum. The missing signals could therefore be assigned from the knowledge of where the deuterioacetate groups were situated. Further assignments were possible using derivatives formed by the deuterioacetylation of known hexa- and hepta-derivatives. It was possible to assign the signals due to the four acetyl groups which had been separated together with tentative assignments of the others.

A complete assignment of the acetyl signals has recently been published by Rathbone.[83] The signals in the ^1H n.m.r. spectrum are very solvent dependent. The chemical shifts of signals can vary quite considerably from solvent to solvent with the relative position of the signals able to change as well.

The spectrum of sucrose octaacetate was recorded in several different solvents. None of these gave sufficiently good separation to allow identification of all the acetate signals. In d_5-pyridine the high field signals were poorly separated whereas in d_6-benzene it was the low field signals that had insufficient resolution. A 1:1 mixture of the two solvents gave baseline resolution of all eight of the acetate signals. It was then possible to assign all eight of the peaks by recording the spectra of several known derivatives which had been perdeuterioacetylated.

Some dependence of the spectrum on the sample concentration was found with small shifts in the chemical shifts of the signals. No change in the relative position of the signals was however observed.

Lee *et. al.* used decoupling techniques and n.m.r. shifts induced by the chemical shift reagents, Eu(fod)3 and Pr(fod)3 to

assign completely the signals in both the ^{1}H and the ^{13}C n.m.r. spectra of some acyl gluco- and galactopyranosides.[84]

The complete assignment of the ^{1}H and ^{13}C n.m.r. spectra of sucrose octaacetate was undertaken by Nishida, Enzell and Morris who used 2-dimensional n.m.r. techniques using both homo- and hetero-nuclear correlations.[85] They found that the assignment was not possible using the 2D-INADEQUATE technique due to strong coupling in the ^{13}C n.m.r. satellite spectra. The assignments were therefore made using a combination of the correlation techniques. A COSY experiment was used to identify the sugar protons although a second stage of coherence transfer was required to identify the signals due to H-5, H-5' and H-6'.[86] The signals in the ^{13}C n.m.r. spectrum were then easily identified from a heteronuclear shift correlation.

To assign unambiguously the signals of the eight acetate peaks required a more complex experiment. The ^{13}C n.m.r. carbonyl resonances of the acetate signals were correlated to the sugar ring proton resonances *via* a three bond coupling. A second correlation was made between the ^{13}C n.m.r. carbonyl resonances and the acetate methyl proton resonances *via* two bond couplings. This long range heteronuclear shift correlation experiment allows the ^{13}C n.m.r. carbonyl signals to be identified from their correlations with the ring protons. The identity of the carbonyl resonances can then be used to identify the acetate signals. Complete assignment of the signals was not possible due to the overlap of two of the acetate signals in the solvent used.

The ^{13}C n.m.r. carbonyl resonances of various peracetylated mono and oligosaccharides have been assigned

using similar techniques in an attempt to establish the existence of any information which might be useful in determining the structure of oligosaccharides.[87] All the other n.m.r. signals were identified as part of the study and ^{13}C enrichment was unnecessary to get a full assignment.

An alternative technique for the assignment of the carbonyl resonances of carbohydrate acetates has been used by Buchanan *et. al.* to assign the signals for β-cellobiose octaacetate and cellulose triacetate.[88] This technique is known as INAPT (Insensitive Nuclei Assigned by Polarisation Transfer). Each of the ^{13}C n.m.r. carbonyl signals can be assigned by polarisation transfer from each of the sugar ring protons in turn. This technique overcomes the lack of sensitivity which can make ^{13}C enrichment necessary for a ^{1}H-^{13}C heteronuclear shift correlation experiment to be successful. Each individual assignment of a signal does however require a separate INAPT experiment.

The ^{13}C and ^{1}H resonances of the sugar ring signals and the ^{13}C resonances of the carbonyl carbon atoms have been assigned for sucrose octabenzoate using a 1:1 mixture of d_5-pyridine and d_6-benzene as solvent.[89] This was done by means of a three bond ^{1}H-^{13}C-shift correlation experiment together with sucrose octabenzoate specifically labelled with ^{13}C in some of the carbonyl groups. By treating the products of partial deacylation of sucrose octabenzoate with an excess of benzyl-*carbonyl*-^{13}C chloride and recording the ^{13}C n.m.r. spectrum the position of deacylation could be identified.

A considerable amount of work on the conformation of sugars has also been carried out particularly by the use of n.m.r. techniques.[90,91] In their paper Binkley *et. al.* comment on the variation in the values of the coupling constant values, in particular the value of J3',4' from those that are expected.[79] The values suggest that the substituents at C-3, C-4, and C-5 adopt quasi equatorial orientations.

Bock and Lemieux used ^{1}H and ^{13}C n.m.r. studies to make a study of the conformation of sucrose in solution.[92] They were interested in the presence of intramolecular hydrogen bonding and found that the hydrogen bond between 1'-OH and O-2 found in the crystal structure is also present in solution. This study suggested that the sucrose molecule is a rigid structure with only slight flexing apparent in the furanose ring.[10] This is in agreement with HSEA calculations.

The use of d_6-dimethylsulphoxide as solvent instead of deuterium oxide caused some changes in the H-3' and H4' coupling constants. Studies of the ^{13}C T_1 relaxation times and isotope effects allowed inferences to be made about the hydroxyl groups. Nuclear Overhauser enhancements were observed to be the same in both deuterium oxide and d_6-dimethylsulphoxide, helping to confirm the theory that sucrose has essentially the same conformation in both solvents. The ^{1}H SIMPLE n.m.r. technique has since been used to show that there is a competitive hydrogen bonding system in solution between OH-1'/O-2 and OH-3'/O-2.[93]

To obtain information about the relative orientation of adjacent residues to one another the use of 2D-J resolved

heteronuclear n.m.r., has been made.[94,95] The information from vicinal couplings in each residue give information on the conformation of the vicinal protons due to a Karplus-type relationship. They do not however give information about the glycosidic linkage. However by studying the long range ^{13}C-^{1}H coupling constants across the linkage the torsional angles can be deduced again *via* a Karplus-type relationship.[96,97,98]

Mass spectrometry has also been a useful technique in the structural determination of sugars. Although the technique is perhaps not quite as useful as n.m.r. spectrometry it is a useful analytical tool. There are three basic ionisation techniques for mass spectrometry. Of these, electron ionisation (EI) is probably the most widely used. This technique has disadvantages when used to record the mass spectra of sugars. The problem is that the ionisation causes too much fragmentation of the sugar. In the mass spectrum of glucose derivatives the substituent at the C-1 position immediately fragments. In the mass spectra of sucrose derivatives it is the glycosidic bond which fragments first.

Although information can be obtained from the fragments formed, particularly if the spectrum of a model compound is available for comparison, it is often not possible to find the parent ion. This information is particularly useful, as, by using accurate mass spectrometry the molecular mass and elemental composition may be determined. These are obviously extremely useful for the identification of a compound.

An alternative means of ionisation is chemical ionisation (CI). This technique is more gentle than EI and so the parent ion

is more often observed. Quite often the mass observed is higher than the molecular ion due to a molecule of the carrier gas being attached to the ion. Fragmentation patterns are also observed using this ionisation technique and are usually very similar to those observed with EI. It can, however, be more difficult to record accurate mass spectra with CI.

The third method is to use fast atom bombardment (FAB) to initiate fragmentation. This is the mildest of the three methods. It can be useful for compounds that do not give good spectra with the other two methods. With acetylated sugars the sequential loss of the acetate groups may be observed before fragmentation of the sugar ring in some cases.

Analysis of the fragmentation pathways of sugar derivatives has been carried out and has included the use of ^{13}C- and ^{2}H-labelled sugars to identify the various ions produced and hence the mechanism of the fragmentations.[99,100] Some information about the structure of a sugar derivative can, therefore, be obtained if the spectrum of a similar structure is known.

The structure of many sugars in the solid state has also been determined by X-ray crystallography.[101,102,103] These include the structure of sucrose octaacetate.[104] Other techniques such as infra-red, ultra-violet and visible spectrometry, optical rotations, melting points and microanalyses are all useful for helping to identify and confirm the structures of sugars and their derivatives.

Chapter Two: The Analysis of Sugar Mixtures.

2.1: Introduction.

The aim of the major part of the work described in this thesis was the enzymatic synthesis of intermediates for the production of sucralose. Since these investigations involve the production of partially protected sugar derivatives, the need for a good analytical system was of extreme importance.

In most of the work described in the introduction, the identification of only the major products, or at best, relative quantities of each class present in a mixture was possible. A "class" is defined here as the group of derivatives of a sugar that all contain the same number of acyl substituents., e.g. mono-, di-, tri-acetates.

Shaw and Klibanov used ^{13}C n.m.r. spectroscopy to identify the position of deacylations.[51] When an acetoxy group is hydrolysed in an acetylated sugar, then the signal in the ^{13}C n.m.r. spectrum due to the carbon atom of sugar ring at the corresponding position changes its chemical shift. This technique has the disadvantages of using the relatively insensitive ^{13}C carbon signals and also only quite simple mixtures of products can be identified with certainty.

^{1}H n.m.r. spectroscopy has also been used extensively to identify the products of reactions. This technique requires the separation of all derivatives including positional isomers. The deacylation at one position will cause several changes in the ^{1}H n.m.r. spectrum. The signal due to the sugar ring proton at the

position of deacylation will move upfield owing to the deshielding effect. There will be corresponding slight shifts in the positions of the other sugar ring signals. Large shifts of the acetyl methyl signals may also be observed. This may result in their relative positions changing, making identification of the methyl signals in the products difficult as these cannot be deduced with reliability from their relative positions in the starting material. However the use of ^1H n.m.r. spectroscopy for the identification of reaction products is useful for the identification of single compounds. For a reaction mixture the shift in the position of the signals, together with the likely overlapping of many of the signals, means that little analysis can be made in this way without complete separation of all the products.

For the sugar of particular interest, sucrose, which contains eight oxygen atoms up to 2^8 different acetylated products are possible and even for the monosaccharide glucose there may be up to 32 (2^5) different products (Fig. 2.1). Complete separation of all the products including the minor ones from a deacylation reaction is likely to be an arduous, if not impossible, task. Hence, time was taken to develop methods of analysis of partially acylated glucosyl esters which did not require complete separation of the products.

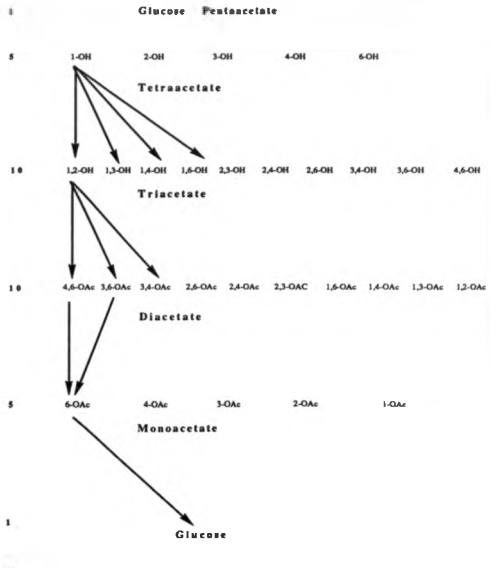


Fig. 2.1: The products from the deacetylation of glucose pentaacetate.

2.2: The development of a method of analysis.

As described above, the analysis of the products of an enzymatic or chemical deacylation reaction is a particularly difficult task. The presence of several very similar hydroxyl groups ensures that regioisomers will have very similar

properties. This makes chromatographic separation of the individual products very difficult. It has been observed, for example, that three of the pentaacetates of sucrose have identical Rf values on chromatography on silica.[39] Given the huge number of possible hydrolysis products of a deacylation reaction there is a likelihood of isomers being impossible to separate. This means that using the techniques described above identification of all the reaction products is not always possible.

Our interest in studying the process of the deacetylations led to the development of an alternative means for the analysis of the products of deacetylation reactions of sugars. This method utilises the techniques of mass spectrometry and n.m.r. spectroscopy to study the products of a reaction in conjunction with chromatography and deuterioacetylation of the samples.

The use of n.m.r. spectroscopy or mass spectrometry on their own is not particularly useful. The ^{1}H n.m.r. spectrum of a mixture containing any more than two or three different compounds is likely to be too complicated to reveal all the information necessary for the identification of the individual components. Even when the components have been separated the analysis can require the use of two dimensional techniques such as the COSY experiment.

In the ^{1}H n.m.r. spectrum of a mixture it is likely that many of the signals due to the sugar ring proton region will overlap, making identification difficult. The same will be true in the acetyl region of the ^{1}H n.m.r. spectrum. ^{13}C n.m.r. spectroscopy has often been used for the identification of different products. It is, however, not likely to be very useful for identifying the components of a mixture of sugar esters

owing to the varying intensities of the signals and the likelihood, again, of overlapping signals. It is also a relatively insensitive technique due to the low relative abundance of the ^{13}C nucleus thus requiring larger quantities or longer acquisition times to produce spectra.

Analysis of a mixture by mass spectrometry will identify the presence of the different classes. For instance it may confirm that sucrose octaacetate has been deacetylated to a mixture of hepta-, hexa- and pentaacetates. These different classes are likely to have different volatilities and ionisation energies so their peak intensities cannot be correlated to the amounts of each class present in the mixture. Thus, little information on their relative abundances can be deduced.

2.3: The analysis of perdeuterioacetylated mixtures.

Given that certain conditions are satisfied, the combination of these two analytical techniques coupled together with the perdeuterioacetylation technique described below can become a powerful analytical method. If some degree of chromatographic separation is introduced into the analysis then complete analysis of the products of a deacetylation process should be possible. This new analytical technique should allow deacetylation reactions to be followed, and once the conditions have been established for a particular sugar they can be used regardless of the catalyst for the deacetylation or the products which are formed.

2.4: Analysis of perdeuterioacetylated samples by n.m.r.

It has previously been reported that partially deacetylated sugars may be reacetylated using d_6-acetic anhydride in pyridine in order to identify the acetyl methyl signals in the ^1H n.m.r.[81,82] This is possible because the acetate signals, after perdeuterioacetylation, appear at exactly the same chemical shift as in the spectrum of the non-deuteriated peracetate. The relative intensities of these signals will permit the extent of deacetylation to be calculated. Those positions that have been fully deuteriated will not give a signal in the ^1H spectrum.

This technique has been used previously to assign the acetyl signals in the ^1H n.m.r. spectrum of a sugar. In a paper published very recently Rathbone commented that this technique could be used as a means of identifying compounds after assignment of the acetate signals had been made.[83]

As described in the introduction, the assignment of the acetate signals can be made with the use of a 2-D heteronuclear shift correlation n.m.r. experiment. This obviates the need for synthesising several different derivatives, identifying them and perdeuterioacetylating them, to enable the signals to be assigned. The assignments can only be made if all the acetate signals are well resolved, which is a *sine qua non* for this analytical technique.

The acetyl groups in sucrose octaacetate are in very similar environments, hence, their signals have very similar chemical shifts and it is likely that some of them will overlap if a spectrum is recorded in a pure solvent. If the solvent is then

changed different signals may overlap. This solvent dependence of the spectrum means that by varying the solvent, or by carefully mixing two solvents together, it is possible that complete separation of the acetate signals may be achieved. For example the eight acetate signals of sucrose octaacetate may be separated by the use of a 1:1 mixture of pyridine and benzene (Fig. 2.2).

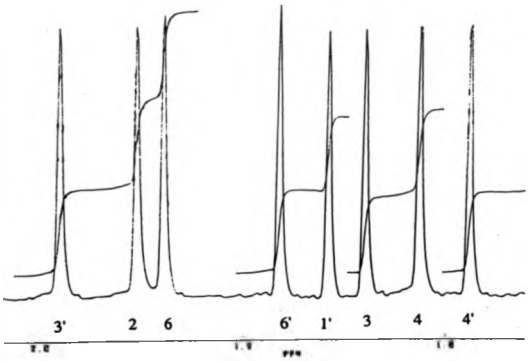


Fig. 2.2: The eight acetate signals in the ^1H n.m.r. spectrum of sucrose octaacetate recorded in d_5-pyridine/d_6-benzene (1:1).

If a mixture of products from the deacetylation of a sugar peracetate is perdeuterioacetylated using d_6-acetic anhydride in pyridine then the product is in effect the same sugar peracetate.

However, it is now substituted by perdeuteriated acetate groups. These deuteriated groups will have replaced each of the acetyl groups which were hydrolysed during the reaction. It is therefore possible to distinguish between those groups which were hydrolysed and those that were resistant to hydrolysis (Fig. 2.3)

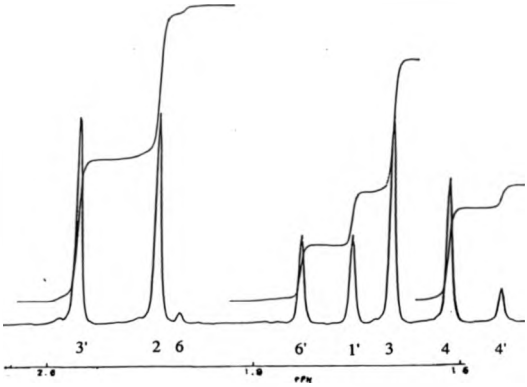


Fig. 2.3: The acetate region of the ^{1}H n.m.r. spectrum of a perdeuterioacetylated mixture of sucrose acetates.

In all other respects the compound is identical to any ordinary sample of the starting material. The ^{1}H n.m.r. spectrum will be identical to that of the starting material in the sugar ring proton region (Fig. 2.4). The ^{13}C n.m.r. spectrum will likewise be

identical. There will however be differences in the acetyl region of the ^1H n.m.r. spectrum. The chemical shift values of all the peaks will be identical regardless of the amount and position of the deuterioacetate groups. Some of the acetate positions will however now contain some perdeuteriated acetate groups. These will not contribute to the ^1H n.m.r. signal corresponding to the acetate group.

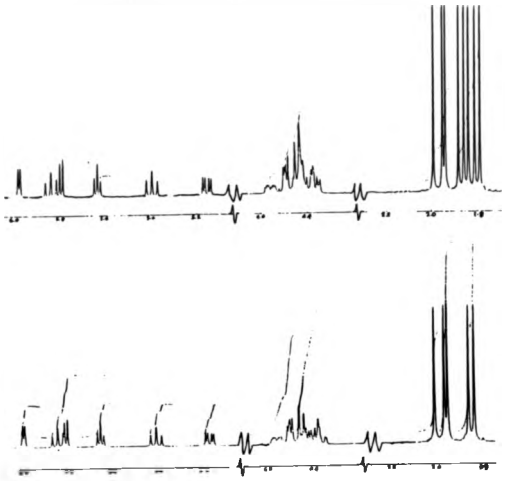


Fig. 2.4: The ^1H n.m.r. spectra of sucrose octaacetate and a perdeuterioacetylated derivative.

The signals due to the acetyl groups which now contain perdeuteriated acetyl groups will therefore be decreased in intensity. This will be by an amount directly proportional to the amount of deuterioacetate at that position. This is the only difference that can be observed between the spectrum of an authentic sample and a sample that contains deuterioacetate.

The outcome is that the relative amount of hydrolysis of each of the individual acetyl group positions can be determined. For example if one position is completely hydrolysed then the corresponding peak in the ^1H n.m.r. spectrum will be completely absent, and if half of the acetate groups at one position have been hydrolysed then the peak corresponding to that acetate will be half the size of a normal peak. As the acetate positions will have been previously identified by the n.m.r. techniques described before, the exact position of the deacetylations can be determined.

2.5: The analysis of perdeuterioacetylated samples by mass spectrometry.

Useful information can also be obtained by the mass spectrometric analysis of a perdeuteriated sample from a deacetylation reaction mixture. The effect of the perdeuterioacetylation is to increase the mass of the deuterioacetylated sugar by three mass units for each perdeuteriated acetate group it contains. For example a sucrose pentaacetate sample which is treated with d_6-acetic anhydride will contain three deuteriated acetate groups (Fig. 2.5). Thus its parent ion in the mass spectrum will be nine mass units greater

than the parent ion obtained from unlabelled sucrose octaacetate.

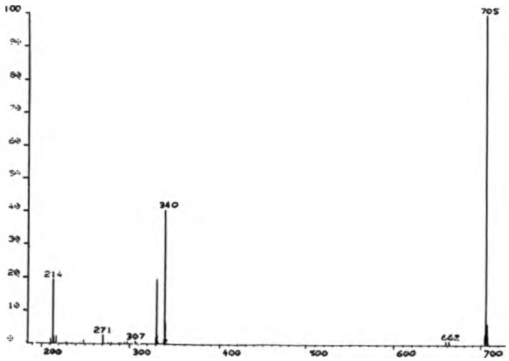


Fig. 2.5: The mass spectrum of a sucrose pentaacetate class after perdeuterioacetylation. M/z=696 for sucrose octaacetate, m/z=705 for a perdeuterioacetylated pentaacetate containing three deuteriated acetyl groups.

If a sample containing a mixture of deacetylation products with varying degrees of acetylation is perdeuterioacetylated and then analysed by mass spectrometry, a series of signals corresponding to the parent ions will be observed. These signals are each separated by three mass units increasing from the parent ion of the unlabelled starting material due to the

increasing content of deuterioacetate groups (Fig. 2.6). The parent ion peak with the highest mass will be due to the perdeuterioacetylated free sugar.

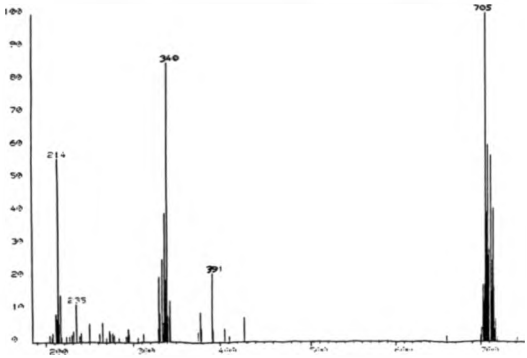


Fig. 2.6: The mass spectrum of a perdeuterioacetylated mixture of sucrose acetates. M/z=696 for sucrose octaacetate increasing by three mass units for each deuteriated acetyl group.

As the mixture contains in effect just one compound, the peracetylated sugar, there will be essentially no difference in the volatilities or ionisation energies of the sample as would be the case with a mixture which had not been treated in this way. This means that the relative intensities of the signals due to each of the acetate classes may be used to determine the

relative composition of the mixture in terms of the classes present. This allows the rate of deacetylation of the starting material, and the production of each of the acetate classes to be followed over the course of the reaction simply by perdeuterioacetylating a sample of the reaction mixture and analysing the mixture by mass spectrometry.

In both glucose and sucrose derivatives the first fragmentation in the mass spectrum is very clear. In glucose pentaacetate and other derivatives such as methyl glucoside tetraacetate, the substituent at the C-1 position is cleaved to give an ion with m/z=331 (Fig. 2.7). If the sample of a mixture of products which has then been perdeuterioacetylated is analysed there will be a series of peaks separated by three mass units in this region.

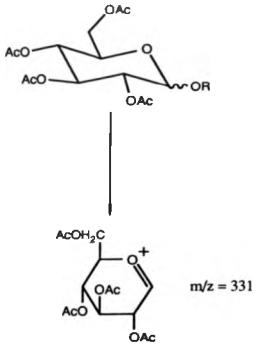


Fig. 2.7: The initial fragmentation of glucose pentaacetate.

In sucrose octaacetate the glycosidic linkage is cleaved to form two fragments (Fig. 2.8). Both the fragment from the pyranose and the fragment from the furanose ring will have a mass of 331 mass units.

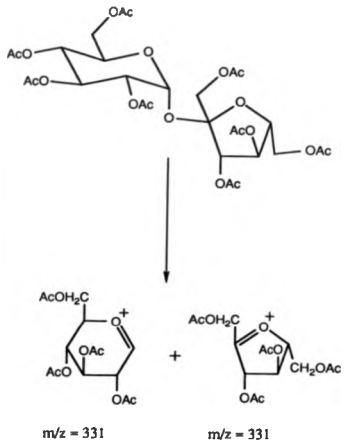


Fig. 2.8: The initial fragmentation of sucrose octaacetate.

Again with a perdeuterioacetylated mixture of sucrose acetates there will be a series of peaks each separated by three mass units. In some cases these peaks can be used to gain some

extra information about the positions of the hydrolysis. For example if the mass spectrum of a hexaacetate contains mainly a peak at 334 mass units this indicates that the major fragments contain one deuterioacetate group. This would indicate that one acetate group in each ring had been hydrolysed. If however peaks were observed at 331 and 337 mass units then both the hydrolysed groups must be from the same ring.

It is also of interest that when sucrose octaacetate fragments in the mass spectrometer, the peak corresponding to the fragment due to the furanose ring is more intense than that due to that of the pyranose fragment. This was observed in the mass spectrum of two octaacetate derivatives, which had been synthesised from two heptaacetates and were then deuterioacetylated to give specifically labelled octaacetate. This led to two peaks one at m/z=331 and one at m/z=334 on fragmentation, corresponding to a fragment with no labelling and to a fragment with one deuterioacetate group. One of the samples had the labelled acetate in the furanose ring and the other had the labelled acetate in the pyranose ring. The peak due to the furanose ring was however larger in both cases, presumably due to a slightly more stable ion fragment. This may be used to give some indication of the positions of deacetylation in certain cases.

2.6: The combination of both techniques to analyse samples.

The analysis of the individual species from a deacetylation mixture is, as described previously, complex. The combination

of the two analytical techniques just described allows a picture of the reaction to be drawn. The positions of deacetylation and the composition of the mixture by class can be determined. However, by combining the use of this type of analysis with the separation of the mixture into classes, a much more detailed analysis of the individual components can be made. The separation of the mixture into classes is relatively simple compared to the separation of each of the individual components. As will be described below, provided that there is some selectivity shown in the deacetylation reaction, a complete analysis of the products may be achieved.

To begin with a deacetylation reaction was followed without including the separation step. This technique is useful for a fast study of a deacetylation reaction although it does not give complete information when used in this form. This technique has been used to study the deacetylation of sucrose octaacetate (Fig. 2.9) stirred as a suspension, and as a solution in acetate buffer solution (pH 5.0). The solubility of sucrose octaacetate in water is very low, but the analysis shows that there is little difference in the reaction kinetics from using the substrate as a suspension. Suspensions were used in the majority of the reactions described in this thesis. The deacetylations were catalysed using a yeast esterase (Glaxo) and were carried out at room temperature. On completion the reactions were stopped by freezing and then dried by lyophilisation. The samples were then perdeuterioacetylated using d_6-acetic anhydride in pyridine and analysed by n.m.r. and mass spectrometry as described before.

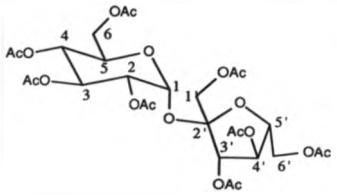


Fig. 2.9: The structure of sucrose octaacetate.

The identification of the 8 acetate peaks of sucrose octaacetate, necessary for this type of analysis, was carried out by Tate and Lyle using the technique of perdeuterioacetylation of known sucrose acetates. This is described in the paper published by Rathbone.[83] We have since been able to confirm the identification of the acetate peaks by use of the ^{1}H-^{13}C shift correlation experiment previously described. This experiment was recorded in a 1:1 mixture of d_5-pyridine and d_6-benzene so that the acetate peaks were separated out.

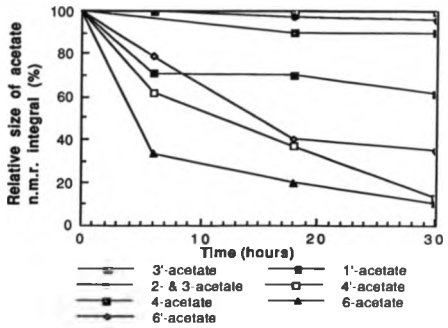


Fig. 2.10: The relative rates of hydolysis of the acetate groups of sucrose octaacetate, catalysed by yeast esterase.

Fig. 2.10 shows a plot of the rate of hydrolysis of the individual acetate positions. The data for this was taken from the integrals of the acetate signals determined from the ^1H n.m.r. spectrum. The percentage hydrolysis of each acetate position is calculated by comparison of the integral of the signal with the integral corresponding to the 3'-acetate group. This position was found to not be hydrolysed throughout the reaction and so provides a reference corresponding to a 100% integral, meaning that it has the same intensity as all the others had at the start of the reaction.

Comparison of this signal with the integral corresponding to a suitable sugar ring proton signal provides an internal standard to confirm the extent of the hydrolysis. In the case of sucrose octaacetate, it was found to be most convenient to use

the integral corresponding to the anomeric proton at C-1 for this purpose, as it was well resolved and the integral easily measured.

The results show that using this particular enzyme two acetyl groups, in addition to the one attached to C-3' which is used an internal standard, are not hydrolysed to any significant degree after a reaction time of 30 hours. These acetyl groups are those attached to C-2 and C-3. If the enzyme selectively hydrolyses the acetate groups so that one or more of them is not hydrolysed at all then the deacetylation study becomes simpler. This would also be true if in a particular sugar one or more of the acetate groups is particularly unreactive. In this case for example with no hydrolysis of three of the groups the number of possible products falls from 256 to just 32. This will be discussed again in a later section.

Four of the acetyl group integrals show the presence of substantial amounts of deuterioacetate indicating that there has been a substantial amount of hydrolysis at these positions during the reaction. Three of these are the primary acetyl groups, that is those attached to C-1', C-6 and C-6'. The other one is the secondary acyl group attached to C-4' which as has been described earlier is often at least as hydrolytically reactive as the primary acetate groups.

After 6 hours approximately 70% of the acetyl groups from C-6 have been hydrolysed with less than 40% of any of the other positions having been hydrolysed. After 18 hours substantial amounts of the acetate groups attached to both C-4' and C-6' have been hydrolysed as well as the C-6 acetate. After 30 hours in excess of 80% of the acetate groups at C-4' and C-6

have been hydrolysed. Mass spectrometric analysis shows that after 6 hours almost 50% of the starting material, sucrose octaacetate, has been hydrolysed, and that by 30 hours it has almost all been hydrolysed (Fig. 2.11).

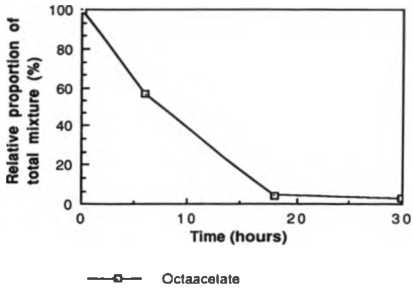


Fig. 2.11: The hydrolysis of sucrose octaacetate.

There is only a small accumulation of the heptaacetate during the reaction, reaching 10% of the total composition after 6 hours (Fig. 2.12). As there is a rapid accumulation of lower acetates this suggests that the heptaacetate is rapidly deacetylated further. The hexaacetate class is present as about 30% of the total composition of the reaction mixture at its maximum after 18 hours, before diminishing as it is further hydrolysed. It can be seen that the pentaacetate concentration is quite high after 18 hours, reaching almost 50% of the mixture after 30 hours (fig. 2.13). This is probably at its maximum before being converted to the tetraacetate.

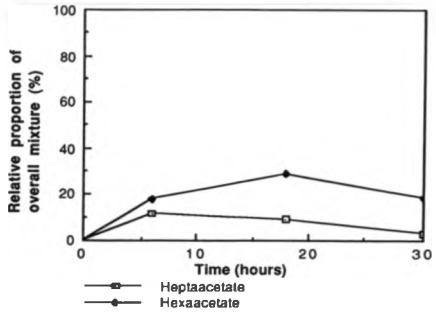


Fig. 2.12: The relative proportions of the heptaacetate and hexaacetate classes.

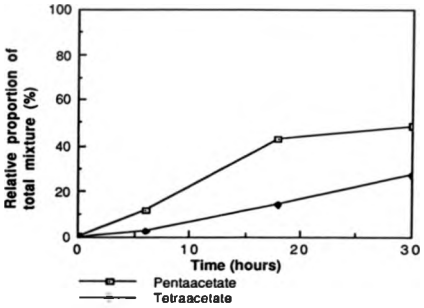


Fig.2.13: The relative proportion of the pentaacetate and tetraacetate classes.

The combination of these results provides a general picture of the reaction. The predominant heptaacetate is likely to be the one with the acetoxy group from C-6 hydrolysed. The hexaacetate class may be more mixed, although as the C-6 and C-4' positions were most rapidly deacetylated, the 1',2,3,3',4,6'-hexaacetate will probably be the major component. The n.m.r. data suggest that the major pentaacetate will be the 1',2,3,3',4-pentaacetate with the 4',6 and 6' acetate groups hydrolysed and the major tetraacetate the 2,3,3',4- derivative. The only triacetate likely to be present is the 2,3,3'- derivative. The data show that a fairly small amount of the acetate at C-4 is deuteriated. This is almost certainly not due to direct enzymatic hydrolysis, but rather due to hydrolysis of the acetyl group at C-6 followed by acetyl migration from C-4 to C-6. As has been described earlier, this is a particular facile migration. The reaction was carried out at pH 5 in order to minimise the amount of acyl migration. However, a small amount still occurs.

There is a limit to the interpretation of these data, which can only be used to get a broad view of this reaction. It can be used as above, to predict when to terminate a reaction in order to get the highest percentage of a particular class of products. It can also be used to make fairly accurate assignments of the major products.

No information can be easily determined about the minor products. It is also more than possible that the composition of a particular class will change markedly over the course of a reaction. For example, the heptaacetate formed first will be the one with the most reactive acetoxy group hydrolysed. This will

then be hydrolysed to a hexaacetate and so on. However, smaller amounts of other heptaacetates may be formed and these may then be further hydrolysed more or less rapidly than the major one. It is thus quite conceivable that a heptaacetate may be formed which is further hydrolysed extremely slowly and so accumulates whilst all the others are being further hydrolysed. It will therefore increase in concentration relative to the other heptaacetates. The type of analysis just described will not be able to identify such a situation whether it occurs in the heptaacetate class or any of the other classes, unless it causes a noticeable effect on the "class picture". To analyze completely the deacetylation a more detailed method is required.

2.7: Analysis of a deacetylation reaction in greater detail: The deacetylation of glucose pentaacetate.

To study the deacetylation process in greater detail, a more advanced version of the above technique was developed by the introduction of a chromatographic separation. As described earlier the separation of all the products of such a reaction will be impossible. However, the products do run in "class" order when subjected to chromatography on silica. This is really to be expected as all the members of a class have the same number of free hydroxyl groups even if the hydroxyl groups are in different positions on the sugar. If the products of a reaction are separated into classes and analysed as described above a considerably larger amount of information can be found. Provided that the reaction being studied has some

degree of selectivity, a total solution of the deacetylation process may be achieved. As some degree of selectivity is required in any deacetylation for it to be of any interest, this method should generally be applicable.

In order to develop this method it was decided to study the deacetylation of β-D-glucose pentaacetate (Fig. 2.14) as a model, using the lipase from *Aspergillus niger*. By starting with a monosaccharide the method could be developed on a reasonably simple process as there are only five acetate groups present . The reaction had been previously studied by Shaw and Klibanov so a comparison of results could be made to verify the use of this method.[52] Shaw and Klibanov's method of analysis was not felt to be sufficiently detailed or straightforward but was able to provide a comparison.

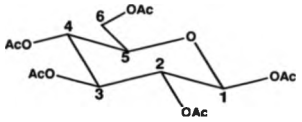


Fig. 2.14: The structure of glucose pentaacetate.

Analysis of a mixture of products from the deacetylation reaction by ^1H n.m.r. spectrometry after perdeuterioacetylation showed that both α- and β-anomers of glucose pentaacetate were present. This occurs because the acetyl group at C-1 is the most reactive towards hydrolysis and this position is therefore rapidly hydrolysed. This means that the sugar now possesses a free hydroxyl group at C-1, the anomeric position. The sugar is

therefore able to epimerise by ring opening and closing to give a mixture of the two anomers (Fig. 2.15).

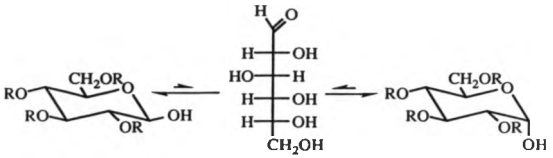


Fig. 2.15: The anomerisation of glucose derivatives.

This complicates the analysis because there are now ten acetyl methyl signals in the ^1H n.m.r. spectrum. Separation of the different signals was not immediately achieved so it was decided that the study of a sugar blocked at C-1 would be more straightforward. The sugar chosen for this was *O*-methyl-α-D-glucopyranoside tetraacetate, a glucose derivative which has four acetate groups, at C-2, 3, 4 and 6 and a methyl group attached to O-1 (Fig. 2.16). It was expected that this methyl group would be stable under the reaction conditions and would therefore not allow anomerisation to occur. The presence of only four acetates should also simplify the analysis.

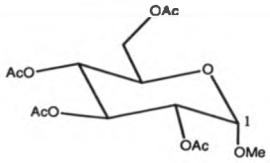


Fig. 2.16: Methyl-α-D-glucopyranoside.

The deacetylation of the methyl glucopyranoside tetraacetate with *A. niger* lipase was carried out and a sample of the reaction mixture was perdeuterioacetylated and analysed by ^1H n.m.r. spectroscopy. The acetate region of the spectrum contained more signals than the four that were expected after perdeuterioacetylation. Analysis of the full spectrum revealed the presence of three different compounds. These were identified as the methyl glucoside as expected together with both α- and β-glucose pentaacetate. This indicated that the anomeric position had once again been deprotected to leave a free hydroxyl group. Anomerisation therefore occurred once more. The products that had not been deprotected at the anomeric position were peracetylated to give the starting material. Those where the C-1 position had been deprotected were peracetylated to give a mixture of the anomers of glucose pentaacetate.

The other anomer of methyl glucoside was also used for a deacetylation reaction. This sugar was also deprotected at the C-1 position although not as quickly as the first anomer. However, this compound was not suitable for our method of analysis

either, because it again gave rise to twelve different signals in the acetyl region of the ^1H n.m.r. spectrum.

Tests on the stability of the starting glucosides under the reaction conditions, but without the presence of the enzyme showed no deprotection at C-1. This suggests that the process was enzymatically catalysed. A solution of the free sugar, methyl-α-D-glucopyranoside, was also stirred with the enzyme in buffer solution. Peracetylation of the product after a reaction time of 18 hours showed the presence of approximately 10% of glucose pentaacetate as well as the starting compound. A control experiment carried out without enzyme showed no removal of the methyl group. The enzyme preparation used was not further purified after purchase and almost certainly contains other activities. It is likely that one of these catalyses the deprotection at the C-1 position.

The use of methyl glucopyranoside was obviously not a suitable case to study for these reasons. Although it would probably have been possible to have found a sugar suitably protected at the C-1 position perhaps by a bulkier group, this was not attempted because further investigations showed that the signals in the acetyl region of the ^1H n.m.r. spectrum of both glucose pentaacetates could be successfully resolved. This meant that the analysis of the hydrolysis of glucose pentaacetate could now be attempted and comparison with the work of Shaw and Klibanov could be made.[52]

The separation was achieved by careful mixing of n.m.r. solvents. It was found that a 1:1 mixture of d_5-pyridine and d_6-benzene gave reasonable separation of the acetate peaks. This was inadequate for the purposes of the experiment as two of

the peaks overlapped enough to make measurement of the integrals inaccurate. On addition of a small amount of d_5-pyridine these two peaks separated sufficiently without any of the others overlapping (Fig. 2.17). It was found that a ratio of d_5-pyridine to d_6-benzene of 7:6 provided the correct solvent to ensure peak separation.

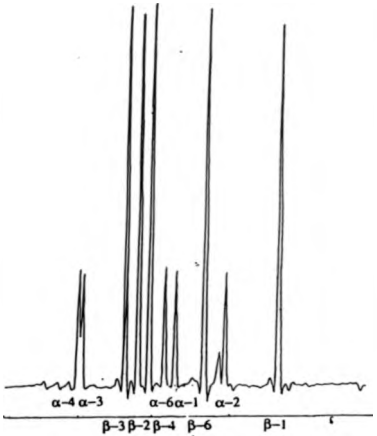


Fig. 2.17: The acetate region of the ^1H n.m.r. spectrum of glucose pentaacetate, showing the ten resolved acetate signals, recorded in a 7:6 mixture of d_5-pyridine and d_6-benzene.

Once separation of all the acetate peaks had been achieved it was necessary to assign each of the signals. The identification of the signals corresponding to each of the

anomers was easily determined by recording the ^1H spectrum of a sample containing a 2:1 mixture of the two anomers of glucose pentaacetate in the 7:6 mixture of d_5-pyridine and d_6-benzene.

To determine the identity of the individual acetate signals the ^1H-^{13}C shift correlation experiment described earlier was used (Fig. 2.18). It was found that the position of the signals in the acetyl region of the ^1H spectrum for each of the anomers was almost identical whether it was recorded on its own or in the presence of the other anomer. This meant that the 2-D correlation could be recorded on the individual anomers. This experiment was carried out for glucose pentaacetate and the acetyl signals were identified.

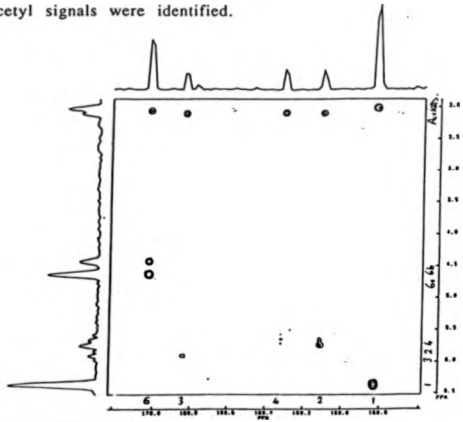


Fig. 2.18a: The 2-D shift correlation spectra used to identify the individual acetate signals in β-D-glucose pentaacetate.

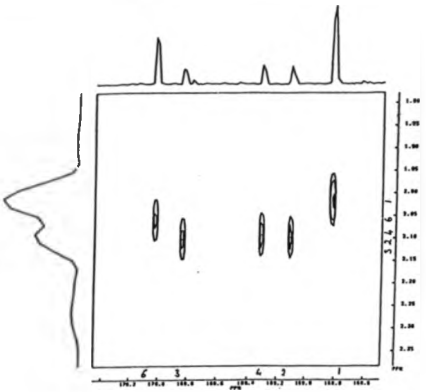


Fig. 2.18b: An expansion of the acetate region of the 2-D shift correlation spectra used to identify the individual acetate signals in β-D-glucose pentaacetate.

The analysis of the deacetylation of β-D-glucose pentaacetate catalysed by lipase A from *A. niger* was then carried out. The experiments were carried out at pH 7.9 in phosphate buffer at 30°C with stirring. Initially, owing to its insolubility, the substrate formed a suspension. As the deacetylation proceeded the suspension dispersed. This was due to the formation of the much more water-soluble lower acetates.

On completion, the reaction mixture was immediately frozen to prevent further reaction and then dried by lyophilisation. A small sample was removed from the mixture; this was used to obtain an overall picture of the deacylation in

the same way as for the wheat germ lipase/sucrose octaacetate experiment. To do this, the sample was perdeuterioacetylated and then analysed by mass spectroscopy. After perdeuterioacetylation the sample will contain glucose pentaacetate with varying amounts of deuterioacetate present. The differing amounts of deuteriation will be revealed as a series of peaks in the mass spectrum separated by 3 mass units i.e. M, M+3, M+6, etc. The relative intensities of these peaks allows the amount of each class of partly acetylated product to be calculated. This can be used to construct an overall picture of the reaction.

In chemical ionisation mass spectrometry with ammonia as the carrier gas, the molecular ions all appear as quasimolecular $(M+NH_4)^+$ ions. The peak for the starting material therefore appears at m/z=408 with the peaks increasing up to m/z=423 for perdeuterioacetylated glucose (Fig. 2.19) The relative intensities of these peaks allows the composition of the mixture to be determined for each reaction time.

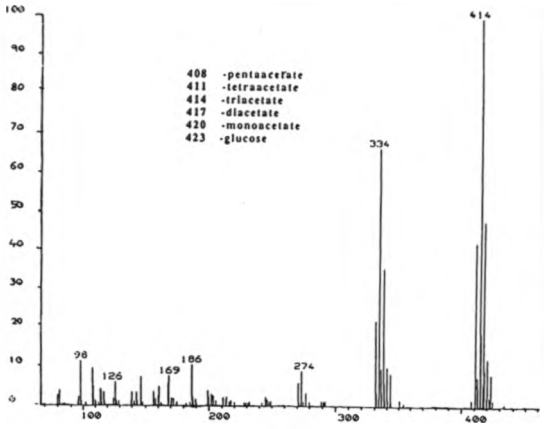


Fig. 2.19: The mass spectrum of a mixture of glucose acetates after perdeuterioacetylation.

The dried product was extracted with a combination of ethyl acetate and ethanol and adsorbed onto a small amount of silica. The mixture was then separated into acetate classes by chromatography on silica gel. Each of the classes of acetates was perdeuterioacetylated using the usual method. The samples were then analysed by ^1H n.m.r. spectroscopy to determine the composition. Mass spectroscopy was also used to confirm the identification of the classes.

As described earlier in this chapter the reduction in size of the integral of an acetate peak corresponds to the amount of

perdeuterioacetylation at a particular position. This is entirely dependent on the degree of hydrolysis at a particular position. One advantage of the presence of both anomers in the samples is that in the ^1H n.m.r. spectrum of the acetyl region two sets of data, one for each anomer, may be obtained for each sample (Fig. 2.20).

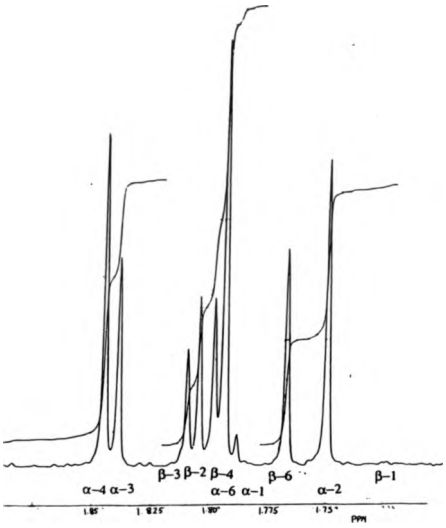


Fig. 2.20: The acetate region of the ^1H n.m.r. spectrum of a perdeuterioacetylated mixture of glucose acetates.

The advantage of analysing the deacetylation mixture after chromatographic separation is as follows. A sample of the hydrolysis reaction will contain a mixture of pentaacetate, tetraacetates, triacetates, diacetates, monoacetates and glucose. As described above the relative composition of the reaction mixture can be determined from the small sample removed prior to the separation. The ^1H n.m.r. spectra of each of the classes can be used to calculate its composition. This is because a series of equations can be derived, relating each of the n.m.r. integrals and the different components of the class to each other. Solution of these equations allows the relative amount of an individual species to be determined as described below.

2.7.1: Analysis of the tetraacetate fraction.

The tetraacetate fraction, after perdeuterioacetylation, contains glucose pentaacetate with all the hydrolysed acetyl groups now replaced by a d_3-acetyl group. The ^1H n.m.r. spectrum of the sample will be identical to that of glucose pentaacetate except that the signals corresponding to the acetyl groups which were hydrolysed during the reaction will be diminished in area.

There are five possible tetraacetates formed in the hydrolysis:- 1,2,3,4-tetraacetyl glucose, 1,2,3,6-tetraacetyl glucose, 1,2,4,6-tetraacetyl glucose, 1,3,4,6-tetraacetyl glucose and 2,3,4,6-tetraacetyl glucose. These are represented in the array below, where p_1 represents the 1,2,3,4-tetraacetate, p_2 the 1,2,3,6-tetraacetate etc.

1	2	3	4	-	p1
1	2	3	-	6	p2
1	2	-	4	6	p3
1	-	3	4	6	p4
-	2	3	4	6	p5

The relative decrease in the signals allows the composition of the class to be calculated. The integral corresponding to the C-1 acetate group has contributions from four of these products, p_1, p_2, p_3 and p_4. An equation can therefore be derived:

$$I_1 = p_1+p_2+p_3+p_4. \qquad 1$$

If the reaction is selective so that one of the groups is not hydrolysed at all, then an equation can be derived for the integral corresponding to this position. It is in effect the same size as the remaining positions were at the start of the reaction and can be denoted by $I_{100\%}$. Alternatively, if there are no positions which have not been hydrolysed, comparison with the sugar ring proton integrals will allow this equation to be derived.

$$I_{100\%} = p_1+p_2+p_3+p_4+p_5 \qquad 2$$

subtracting 1 from 2 gives:

$$I_{100\%}-I_1 = p_5 = d_1 \qquad 3$$

where d_1 corresponds to the decrease in the size of the integral corresponding to the acetate group at C-1.

Therefore the percentage of the 2,3,4,6-tetraacetate can be determined from the percentage decrease in the peak corresponding to the C-1-acetyl group which is the group hydrolysed to form this tetraacetate. Similarly the percentage of 1,3,4,6-tetraacetate is given by the decrease in the peak corresponding to the C-2-acetyl group.

2.7.2: Analysis of the triacetate fraction.

In this case there are ten possible products each containing three acetate groups as shown in the array below. The appearance of a number indicates that the corresponding acetyl group is present in the particular product.

1	2	3	-	-	p1
1	2	-	4	-	p2
1	2	-	-	6	p3
1	-	3	4	-	p4
1	-	3	-	6	p5
1	-	-	4	6	p6
-	2	3	4	-	p7
-	2	3	-	6	p8
-	2	-	4	6	p9
-	-	3	4	6	p10

Using the above array a series of equations relating the integrals of each acetate peak to the individual triacetate species can be derived. The relative size of the integral corresponding to a particular acetate group will depend on the quantities of the species that contribute towards it. If every different product in the mixture contains a particular acetate group (i.e. the group is totally resistant towards hydrolysis) then the integral will have a contribution from all the products and so will be equivalent in size to the integral at the start of the reaction ($I_{100\%}$). If there is some hydrolysis of the position, then the integral will have contributions from the products which contain that acetate. At the same time the decrease in the size of the integral is due to all the products which do not contain that acetate group. For example the decreases in the signal of the C1-acetyl, denoted by d_1, is due to the possible formation of four products. These are indicated in the above array by p_7, p_8, p_9 and p_{10}. None of these products will contain an acetate at C-1 and so will make no contribution to the corresponding integral. An equation can therefore be formed relating the decrease in the integral to the relative amounts of these four products:

$$d_1 = p_7 + p_8 + p_9 + p_{10}$$

In the same way a similar equation can be derived for the decrease in size of the integral corresponding to the C-2 acetate. In this case the four products not containing an acetate at C-2 are p_4, p_5, p_6 and p_{10}. This gives:

$$d_2 = p_4+p_5+p_6+p_{10}$$

Similar expressions can be derived for each of the other acetyl positions.

$$d_3 = p_2+p_3+p_6+p_9$$
$$d_4 = p_1+p_3+p_5+p_8$$
$$d_5 = p_1+p_2+p_4+p_7$$

This gives us 5 equations with 10 unknowns. However given some selectivity in the deacetylation reaction these can be simplified. In this case the acetate group at C-6 is resistant to hydrolysis and as seen in the tetraacetate class the acetate group at C-1 is totally hydrolysed before the others. The resistance of the C-6 acetate group to hydrolysis can be checked by comparison of its ^1H n.m.r. signal with those of the sugar ring protons. This means that all the products must contain an acetate at C-6, which eliminates the products represented by p_1, p_2, p_4 and p_7 and that the product must not contain an acetate at C-1, eliminating products p_1-p_6. Eliminating these products from the above equations simplifies the problem to four equations and three unknowns which can be solved.

$$d_1 = p_8 + p_9 + p_{10}$$
$$d_2 = p_{10}$$
$$d_3 = p_9$$
$$d_4 = p_8$$

Values for the relative amounts of each of the triacetates can therefore be determined from the n.m.r. data. This can be used in conjunction with the previously described analysis of the

composition of the reaction mixture to calculate the proportion of each individual triacetate species in the overall mixture.

2.7.3: Analysis of the diacetate fraction.

As with the triacetate class the diacetates must contain an acetate group at C-6 and must not contain one at C-1. In this case, of the 10 possible diacetates, only 4 contain the acetate at the C-6 position. As the C-6 position is resistant to hydrolysis, all of the products will contain an acetate group at this position. The possible products are shown by the array below :-

1	-	-	-	6	p1
-	2	-	-	6	p2
-	-	3	-	6	p3
-	-	-	4	6	p4

This array allows a set of expressions relating the n.m.r. integrals to the acetate species to be derived leading to four equations and four unknowns:-

$$d1 = p2 + p3 + p4$$
$$d2 = p1 + p3 + p4$$
$$d3 = p1 + p2 + p4$$
$$d4 = p1 + p2 + p3$$

The relative proportions of the diacetates can therefore be calculated by the solution of these simultaneous equations.

The monoacetate fraction consists almost entirely of 6-*O*-acetyl glucose and the relative amount of this monoacetate and of glucose may simply be determined from the mass spectrometric data used to determine the class composition of the total mixture.

2.7.4: Results.

The analysis of the overall reaction is determined from the mass spectrometric data found from the perdeuterioacetylated samples removed prior to the separation process. Sets of data were determined from the samples removed from two separate experiments. The graphs obtained from plots of the data are very similar in pattern. The differences that are present indicate that the second experiment proceeded slightly faster than the first probably due to a stronger buffer solution (Figs. 21a, 21b). The data shows that the complete hydrolysis of the starting material, glucose pentaacetate, had occurred after 75 minutes, although in excess of 80% had been hydrolysed after just 40 minutes of reaction (fig. 2.21a). This follows the pattern expected for the deacetylation of a substrate, showing a decaying concentration over the course of the reaction.

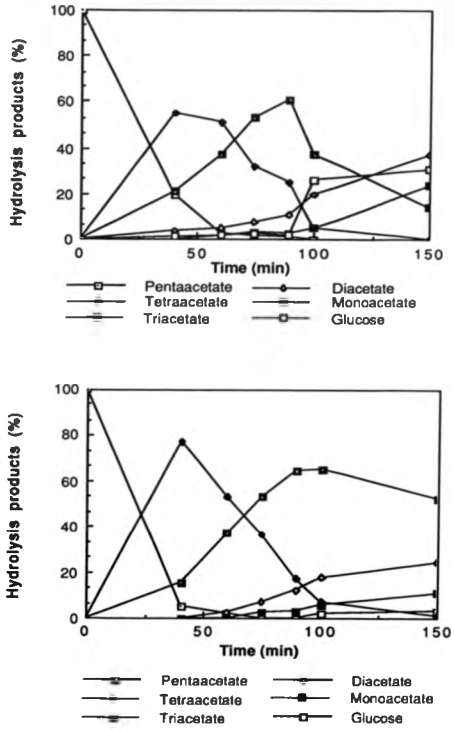


Fig. 2.21a & b: The composition of the mixture by acetate class, from two separate experiments, for the deacetylation of glucose pentaacetate catalysed by lipase A.

The tetraacetate accumulated rapidly, reaching maximum concentration after approximately 40 minutes at which time it formed almost 80% of the reaction mixture. From this time onwards it decreased in quantity as it was further deacetylated to form the triacetates, until after 100 minutes there was only a small amount left. After 150 minutes all the tetraacetate class had been further hydrolysed.

The triacetate class had its maximum composition at between 90 and 100 minutes. At this point it accounted for about 65% of the total mixture. This again was further deacetylated to form the diacetate class, so that after 150 minutes it was only present in a very small amount.

The diacetate class accumulated slowly over the course of the reaction. It was first apparent after 60 minutes and slowly increased in concentration until after 150 minutes it became the major component of the mixture.

The relative quantities of both the monoacetate and of glucose were increasing towards the end of the reaction as would be expected. The resistance of the C-6 acetate to hydrolysis mentioned earlier should have ensured that the monoacetate to glucose conversion was fairly slow. There is a reasonably high proportion of glucose present suggesting that the hydrolysis of the C-6 acetate is more favourable in the absence of other acetate groups.

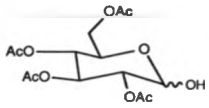


Fig. 2.22: The major tetraacetate: 2,3,4,6-tetra-*O*-acetyl-D-glucose.

The analysis of the tetraacetate group is, as mentioned earlier, straightforward. Of the five possible products, the data obtained from the n.m.r. integrals of the acetyl signals, indicated the presence of just three. One of these, the 2,3,4,6-tetraacetate (Fig. 2.22) was present in a substantially greater concentration than the others. By combining the data about the tetraacetate class composition with the data detailing the contribution of the tetraacetate class to the overall reaction mixture the absolute amount of the individual tetraacetate species could be calculated. This data is shown in fig. 2.23. This shows that the 2,3,4,6-tetraacetate accounts for approximately 50-55% of the mixture between 40 and 60 minutes. The minor products, the 1,3,4,6- and 1,2,4,6-tetraacetates accounted for, at most, 5% of the total mixture.

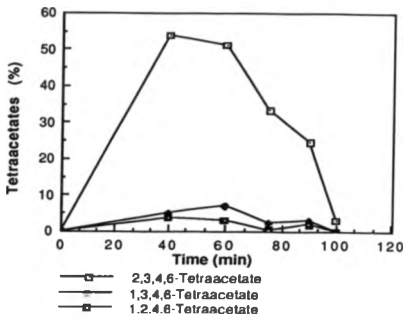


Fig. 2.23: The composition of the tetraacetate class.

The triacetate composition can be straightforwardly determined from the analysis of the n.m.r. integrals. This class was not dominated by any one particular species. Once again in combination with the data for the overall composition of the mixture the absolute amount of each of the triacetates could be calculated. This data is shown in fig. 2.25. There were three different triacetates present in the mixture, all had a peak concentration at about 90 minutes with the major one, the 2,4,6-triacetate, accounting for 25% of the total product (Fig. 2.24).

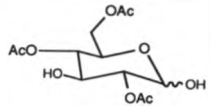


Fig. 2.24: The major triacetate: 2,4,6-tri-*O*-acetyl-D-glucose.

The other two, the 3,4,6- and 2,3,6-triacetate accounted for 19% and 15% respectively. As both the two major triacetates involved no deacetylation of the C-4 acetyl group it would seem to suggest that this group is most resistant towards hydrolysis after the C-6 group.

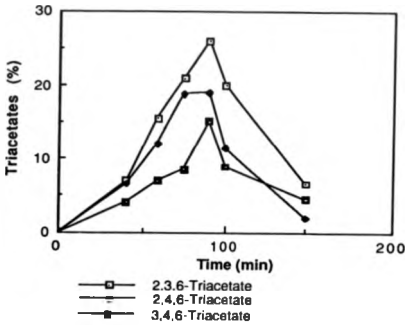


Fig. 2.25: The composition of the triacetate class.

Analysis of the diacetate fraction, using the methods described above, reveals the presence of three diacetate compounds (fig. 2.26). One of these, the 2,6-diacetate, is present in higher concentration than the other two, accounting for over 20% of the total product after 150 minutes. This diacetate together with the 3,6-diacetate increase by a large amount between 75 and 150 minutes. The third diacetate observed, the 4,6-diacetate, remains at an almost constant concentration of just under 5% for this period. This suggests that this particular diacetate was either rapidly hydrolysed to the monoacetate or alternatively only formed by an unfavourable hydrolysis. The selectivity therefore seemed to have changed from that detected for the triacetates as the two preferred diacetates were the 2,6- and the 3,6-derivatives. This means that the C-4 acetate must have been more reactive towards hydrolysis in the 2,4,6-triacetate than it was in the tetraacetate derivative. Occasionally a diacetate class sample was contaminated by some of the monoacetate, detected by the mass spectrometric analysis of the samples and allowances for the amount of contamination were made.

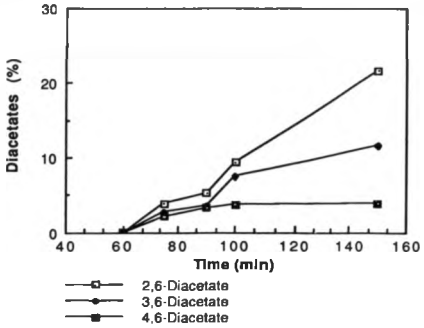


Fig. 2.26: The composition of the diacetate class.

As mentioned previously the concentrations of the 6-monoacetate and of glucose are simply determined from the data for the overall mixture. This data is shown in fig. 2.27.

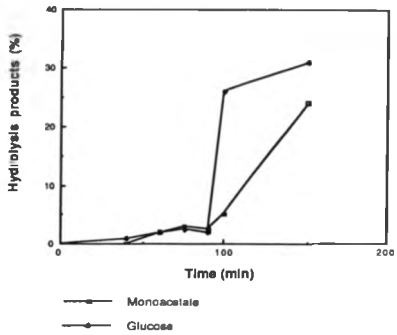


Fig. 2.27: The relative amounts of the monoacetate and of glucose.

A full analysis of the deacetylation of glucose pentaacetate catalysed by *A. niger* lipase has therefore been achieved. It is a particularly interesting deacetylation in that the acetoxy group most resistant to hydrolysis is at the C-6 position. This is the primary position and would be expected to be the most reactive of the groups. This shows the advantages of enzymatic deacetylations. The relative reactivity of enzymatic deacetylation is by no means necessarily the same as would be expected from the chemical reactivities of a compound. The selectivity of a deacylation may vary considerably with different enzymes.

This method of analysis shows similar results to that of Shaw and Klibanov.[52] A considerable amount of extra detail is,

however, provided relating to the deacetylation process. The identification and estimation of the individual species in the potentially complex mixture is possible. The method should also be generally applicable, without modification, to the study of the deacetylation of glucose pentaacetate by any other enzyme provided that a certain minimum selectivity is observed in the system. This selectivity is readily apparent from the analyses of the crude mixtures without any need for separation. This method has been described earlier.

It should be noted that this enzymatic deacetylation reaction was carried out at pH 7.0. At this pH the conditions are suitable for the acetyl migrations previously described to occur. As the C-6 acetate is resistant to hydrolysis, the particularly favourable C-4 to C-6 migration is not possible. It is, however, possible that other migrations around the ring may occur. This analytical method only gives information about the identity of the different products present when the deacetylation reaction is stopped. The enzyme may not necessarily catalyse the formation of these products as rearrangements may occur during the reaction and will not be detected by this method of analysis. The true selectivity of an enzyme can really only be determined when there is no possibility of acetyl migrations occurring.

There are of course errors inherent to this analytical technique. An estimation of the size of these errors could be made for this analysis owing to the presence of the two anomers, which were in equilibrium with one another. Two sets of n.m.r. integral data, one for each anomer, were produced for

each experiment. A comparison of the two sets of data allowed an estimation of the error to be made as ±8%. The analysis of the deacetylation of sucrose octaacetate described below used almost identical procedures and techniques. It is, therefore, assumed that the same errors will be present in the analytical method.

2.8: Deacetylation of sucrose octaacetate using yeast esterase.

To test our method of analysis further the deacetylation of the disaccharide, sucrose octaacetate (Fig. 2.28), catalysed by wheat germ lipase was carried out. The analysis of the deacetylation is more complex this time owing to the increased number of acetate groups present in the substrate. Provided that there is selectivity in the reaction, a complete solution may be possible although the analysis of the penta-, tetra- and triacetate classes is potentially the most difficult because of the number of possible products. If the hydrolysis reaction is selective then a large number of these products may be ruled out, thus simplifying the mathematical analysis considerably.

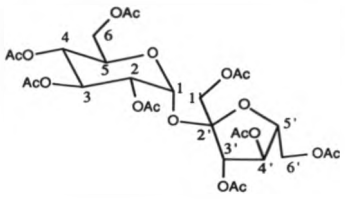


Fig. 2.28: Sucrose octaacetate.

The experimental procedure followed was very similar to that previously employed for the glucose pentaacetate/lipase A analysis. A suspension of sucrose octaacetate was stirred with wheat germ lipase in phosphate buffer (pH 7.0) at 30°C. After a set time the experiment was stopped and the mixture was lyophilised. Initially, the reactions were carried out on a small scale and the product was perdeuterioacetylated without further purification. Analysis of these samples by mass spectrometry allowed a profile of the reaction to be determined which allowed reaction times for the larger samples to be determined (Fig. 2.29).

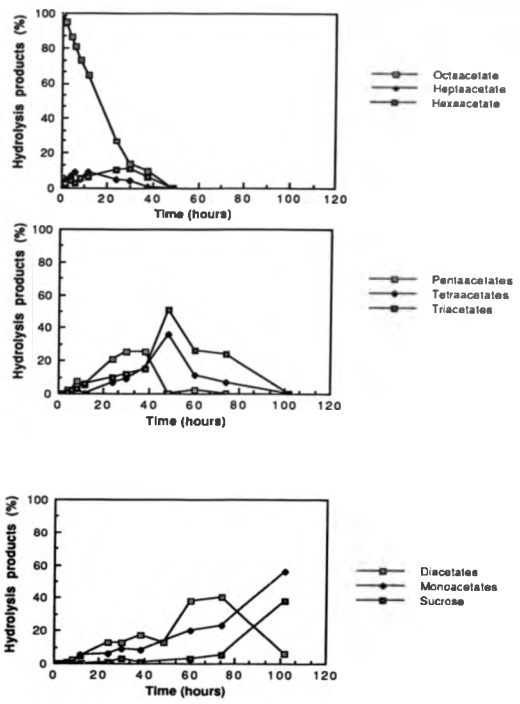


Fig. 2.29: The class composition of the mixture.from the small scale deacetylation of sucrose octaacetate catalysed by wheat germ lipase.

The large scale reactions were carried out in an identical manner. On completion the samples were frozen and lyophilised. After extraction with ethyl acetate and ethanol to remove the majority of the crude enzyme a small sample was removed for analysis of the class composition. The remainder of the mixture was adsorbed onto a small amount of silica gel. The mixture was then separated by chromatography on silica gel, eluting with a combination of petroleum ether (b.p. 40°-60°C), ethyl acetate and ethanol.

The fractions were analysed by t.l.c. and collected into acetate classes. These were then perdeuterioacetylated using d_6-acetic anhydride in pyridine as in the previous analyses. N.m.r. and mass spectrometry were then used to obtain the data necessary for the class analysis. The data from the n.m.r. integrals were used to calculate the composition of the class with the mass spectrometric data confirming the identity of the class.

The overall profile of the reaction shown in fig.2.30 shows that the expected pattern of hydrolysis is found. The substrate, sucrose octaacetate, is hydrolysed to produce the lower acetate classes until after 36 hours it has all been partially deacetylated. The heptaacetate class is the first to be produced from the deacetylation, but is rapidly further hydrolysed. There is very little accumulation of the heptaacetate class with no more than 10% of the total mixture being heptaacetate at any time.

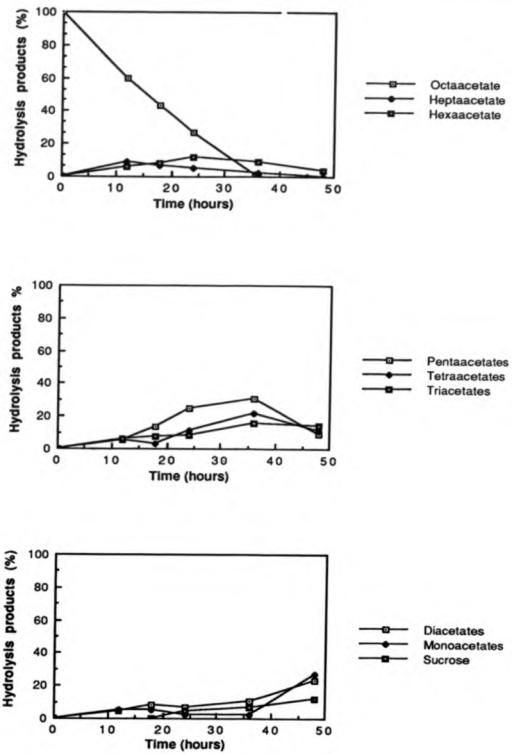


Fig.2.30: The class composition of the large scale deacetylation.

2.8.1: Analysis of the heptaacetate class.

The composition of the heptaacetate class and hence the amount of each of the individual heptaacetate species is straightforward to determine. There are 8 possible heptaacetates which may be formed as shown below.

An array to represent the eight heptaacetate species.

2	3	4	6	1'	3'	4'		p1
2	3	4	6	1'	3'		6'	p2
2	3	4	6	1'		4'	6'	p3
2	3	4	6		3'	4'	6'	p4
2	3	4		1'	3'	4'	6'	p5
2	3		6	1'	3'	4'	6'	p6
2		4	6	1'	3'	4'	6'	p7
	3	4	6	1'	3'	4'	6'	p8

The integral corresponding to the C-2 acetate peak is made up from contributions due to seven of the heptaacetate species

$$I_{C\text{-}2} = p1+p2+p3+p4+p5+p6+p7 \qquad 4$$

Similar equations can be derived for each of the acetate peaks giving eight equations and eight unknowns. This means that the solution may be found. If there is selectivity in the reaction such that one of the acetate groups is not hydrolysed

then this will have a contribution from all the species and so will correspond to

$$I_{C\text{-}100\%} = p1+p2+p3+p4+p5+p6+p7+p8 \qquad 5$$

subtracting 4 from 5 gives

$$I_{C\text{-}100\%} - I_{C\text{-}2} = p8 = d_2 \qquad 6$$

Where d_2 is the decrease in the integral corresponding to the acetate group at C-2.

This value can be determined from comparison of the integral for the C-2 acetate with the integral corresponding to the 100% integral, allowing the proportion of species p8 in the heptaacetate class to be calculated. This process can be repeated for the rest of the heptaacetates to find the class composition. Using this information in conjunction with data about the relative amount of the heptaacetate class in the crude mixture, the amount of each individual heptaacetate species in the overall mixture can be determined.

In this reaction three major heptaacetates were formed i.e. the 1',2,3,3',4,4',6-heptaacetate which was the major component although it only accounted for 3% of the total mixture and the 1',2,3,3',4,6,6'- and the 2,3,3',4,4',6,6'-heptaacetates (fig. 2.31).

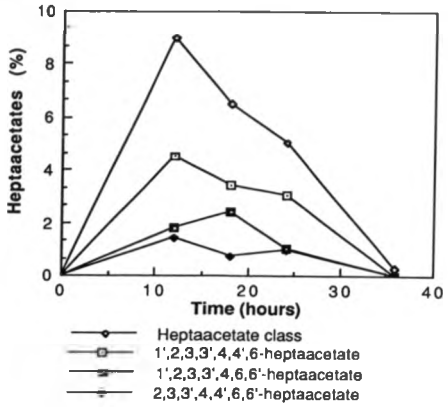


Fig. 2.31: The composition of the heptaacetate class.

2.8.2: Analysis of the hexaacetates.

The hexaacetate class is more complicated to analyse due to the possibility of forming 28 different products. A set of equations similar to those derived for the heptaacetates can be found and the analysis, for simplification, relies on the hydrolysis being selective. The data from the n.m.r. integrals provide 8 equations but this time there are 28 unknown quantities. To achieve a solution some simplification is required. By eliminating all the products which cannot be formed, this simplification can be made. All the hexaacetates that cannot be formed by the subsequent deacetylation of the identified

heptaacetates can be eliminated from the analysis. All the hexaacetates containing acetate groups which have been totally hydrolysed, indicated by complete absence from the ^1H n.m.r spectrum, together with those not containing groups which have not been hydrolysed, indicated by still having a 100% integral in the ^1H n.m.r. spectrum may also be eliminated.

In the sucrose octaacetate/wheat germ lipase hydrolyses the identification of the hexaacetates was simplified by the selectivity of the deacetylation. For example, from the data on the heptaacetates, the hexaacetates had to have at least one of either the C-1', C-4' or C-6' positions deacetylated. This information allows the number of possible products to be substantially reduced. The n.m.r. data for some of the samples also indicated that the C-2, C-3,C-3' and C-6 positions had not been hydrolysed, hence further simplifying the analysis. Provided that there is some selectivity in the hydrolysis, not necessarily as much as above, the analysis can be simplified sufficiently such that the composition may be determined. The hexaacetate class from the 12 hour reaction was the only one for which a complete solution could not be achieved. In all cases one major product, 1',2,3,3',4,6-hexa-*O*-acetyl sucrose, was found (Fig. 2.32).

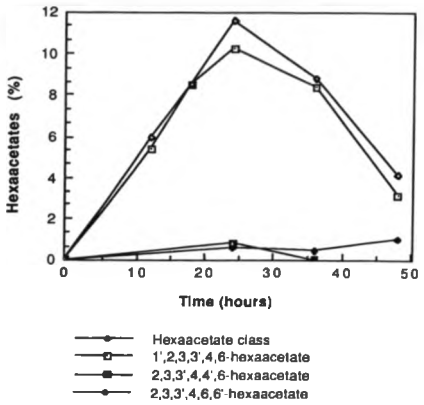


Fig. 2.32: The composition of the hexaacetate class.

The other hexaacetates identified were the 2,3,3',4,4',6- and 2,3,3',4,6,6'-hexaacetates. As with the heptaacetates there was no significant accumulation of any of the hexaacetate species. Between 18 hours and 24 hours the 1',2,3,3',4,6-hexaacetate accounted for just over 10% of the total mixture, with the other species only being present as about 1% of the total mixture.

2.8.3: Analysis of the pentaacetates.

The pentaacetate class may be composed of up to 56 different compounds. To allow complete analysis of the class some selectivity must be seen in the products thereby

simplifying the equations which can be formed for the different peak integrals. In this deacetylation reaction 3 pentaacetates were observed throughout the reaction together with small amounts of 3 other products identified at the end of the reaction (Fig. 2.33).

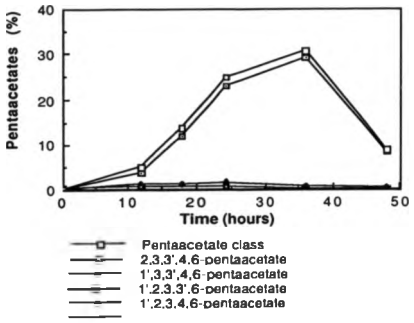


Fig. 2.33: The composition of the pentaacetate class.

One of the pentaacetates in particular did accumulate in the reaction mixture, accounting for 34% of the total products after 48 hours. The other two pentaacetates were only present in quantities of about 15% of the total mixture.

2.8.4: Analysis of the tetraacetates.

The tetraacetate class is potentially the largest with 70 possible species. In this case, the analysis was again simplified by the selectivity shown in the products. The predominance of one particular pentaacetate together with the presence of some groups showing no hydrolysis during the reaction made the analysis much less difficult. This is because many of the theoretically possible products can be eliminated from the calculations.

In the first two samples, three tetraacetates were observed with the 2,3,3',6- and the 3,3',4,6-tetraacetates being the major two. They accounted for 6.3% and 7.2%, respectively, of the total reaction mixture after 18 hours, with the 3,3',4,6-tetraacetate becoming more prevalent after 36 hours accounting for 17.5% of the mixture (Fig. 2.34). This tetraacetate was then further hydrolysed as indicated by the fact that the tetraacetate class, after 48 hours, was small and was composed of two different compounds, the 2,3,3',4- and 2,3,3',6-tetraacetates.

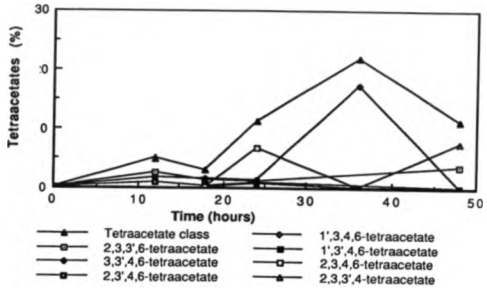


Fig. 2.34: The composition of the tetraacetate class

2.8.5: Analysis of the triacetate class

The triacetate class was analysed in similar fashion. The composition of the class after 18 hours could not be totally determined, but the major product was identified as 2,3,3'-tri-*O*-acetyl sucrose present as 5.4% of the mixture. After 36 hours two major products, the 3,3',6- and 3',4,6-triacetates were present together with a mixture of several minor products. At this point the triacetate class accounted for 15% of the mixture (Fig. 2.35).

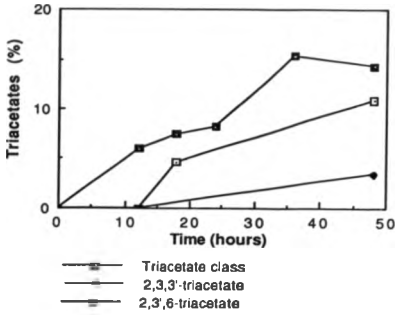


Fig. 2.35: The composition of the triacetate class.

The diacetate class consisted mainly of the 2,3'-diacetate after 18 hours with the 3,3'-diacetate also present. After 48 hours only the 3',6-diacetate was observed (Fig. 2.36). No monoacetate fractions were successfully separated from the mixture.

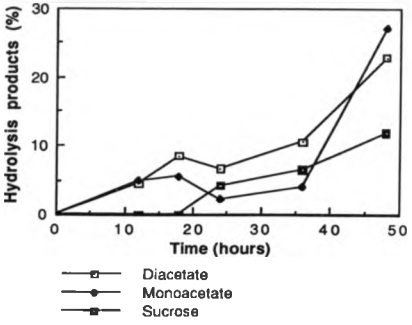


Fig. 2.36: The relative amounts of the diacetate class, monoacetate class and sucrose in the reaction mixture.

2.8.6: Discussion.

The overall reaction profile for the reaction shows the expected results. The sucrose octaacetate decreases in concentration as it is hydrolysed to form heptaacetates. These appear in the first sample taken after the beginning of the reaction and are then further hydrolysed to form hexaacetates. There is no significant accumulation of any of the heptaacetates with none of the individual species accounting for more than 10% of the products.

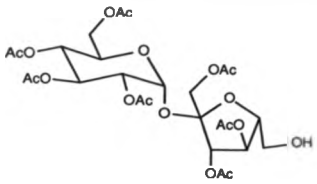


Fig. 2.37: The major heptaacetate: 1',2,3,3',4,4',6-hepta-*O*-acetyl sucrose.

This is to be expected as there are three heptaacetates observed, the 1',2,3,3',4,4',6- (Fig. 2.37), 1',2,3,3',4,6,6'- and 2,3,3',4,4',6,6'-heptaacetates, all of which are present in fairly similar amounts. This suggests that the C-1', C-4' and C-6' positions all have similar reactivities towards hydrolysis by this enzyme. This means that further hydrolysis to hexaacetates is likely to be favourable thereby preventing any build up of this class.

Only one major hexaacetate is observed throughout the reaction which is 1',2,3,3',4,6-hexa-*O*-acetyl sucrose (Fig. 2.38) This species will be formed by deacetylation of both the 1',2,3,3',4,4',6- and 1',2,3,3',4,6,6'-heptaacetates. This hexaacetate accumulates to form about 10% of the total mixture. There is no other hexaacetate apparent in any quantity, with small amounts of the 2,3,3',4,4',6- and 2,3,3',4,6,6'-hexaacetates being observed. These are presumably formed by further deacetylation of the other heptaacetate observed.

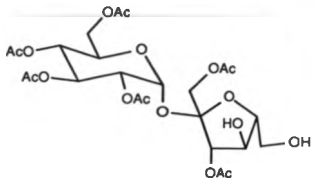


Fig. 2.38: The major hexaacetate: 1',2,3,3',4,6-hexa-*O*-acetyl sucrose.

In the pentaacetate class there is again one major species, 2,3,3',4,6-penta-*O*-acetyl sucrose (Fig. 2.39) which is the first product to be observed in a significant amount. After 48 hours this product is present as 34% of the total mixture. This product will be formed by deacetylation of both the major hexaacetate and the two minor ones. It is by far the most prominent species suggesting again that the C-1', C-4' and C-6' acetates are the most easily hydrolysed groups.

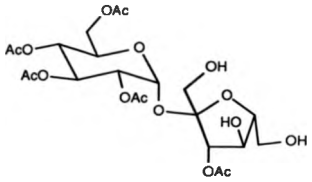


Fig. 2.39: The major pentaacetate: 2,3,3',4,6-penta-*O*-acetyl sucrose.

The two minor pentaacetates observed throughout the reaction are 1',3,3',4,6- and 1',2,3,3',6-penta-*O*-acetyl sucrose. In both of these products one of the acetate groups in the pyranose ring has been hydrolysed, together with the groups at C-4' and C-6' in the furanose ring. The two groups that are deacetylated in the pyranose ring, those at C-2 and C-4, are both at secondary positions. The primary C-6 acetate group is not hydrolysed in any of the pentaacetate species, indicating a surprising lack of reactivity. In the last fraction analysed, two other pentaacetate species are observed. They are both minor products and may be slowly accumulating throughout the reaction, possibly because they are relatively stable to further hydrolysis.

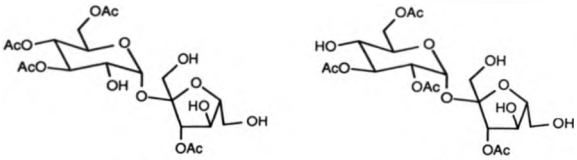


Fig. 2.40: The two major tetraacetates: (a) 3,3',4,6- and (b) 2,3,3',6-tetra-*O*-acetyl sucrose.

The tetraacetate fraction is a considerably more mixed class. Three tetraacetates, 2,3,3',6-, 3,3',4,6- and 2,3',4,6-tetra-*O*-acetyl sucrose, are observed throughout the reaction with the first two of these accumulating to 6.3% and 7.2%, respectively, after 18 hours (Fig. 2.40). All three are formed by deacetylation

of one of the pyranose ring positions in the major pentaacetate. Again it is the secondary positions that are hydrolysed in the presence of a primary acetate group. As observed in the pentaacetate class the C-2 and C-4 positions are more readily hydrolysed than the C-3 position. Several other minor tetraacetate species are observed in the later samples. The most predominant of these is the tetraacetate with all the furanose ring acetates hydrolysed.

The fact that this class is more mixed shows that the C-1', C-4' and C-6' positions are far more reactive than the other positions towards hydrolysis with this enzyme. Once these groups have been hydrolysed there is a loss of selectivity due to the comparable reactivities of, in particular, three of the remaining groups, C-2, C-3 and C-4. Their similar reactivities cause a mixture of products to be formed with none predominating.

The triacetate class is again a mixed class. Four species in all are observed, all but one of them still possessing an acetate group at both C-6 and C-3'. This would appear to confirm the comparable reactivities of the C-2, C-3 and C-4 positions.

The chromatography of the diacetate classes was not successful with mixtures of diacetates and monoacetates being collected together with poor separation. This made it impossible to calculate the class compositions. Those species which were identified all possessed the C-3' acetate group, although, in other mixtures there had been some hydrolysis of this position. This was identified by comparison with the sugar ring proton integrals. The major monoacetate would, however, be expected

to be the 3'-monoacetate. Total hydrolysis to give sucrose is observed so there is no totally unreactive position.

The overall analysis of the reaction suggests the presence of three particularly reactive acetyl groups, C-1', C-4' and C-6', three positions which are fairly easily hydrolysed, C-2, C-3 and C-4 and two groups which are fairly unreactive, C-6 and in particular C-3'. The apparent order of reactivity is:

C-6' > C-4' > C-1' >> C-4 > C-2 > C-3 >> C-6 > C-3'

This is another example of the C-4' position showing comparable reactivity to the primary C-1' and C-6' positions. The C-6 primary position is seen to be remarkably unreactive, with all but one of the secondary positions being preferentially hydrolysed. This is very different from the observed chemical reactivity of the sugar and shows the potential that biotransformations have for producing partially protected sugars which cannot easily be made by chemical methods.

Chapter Three: The Synthesis of N-Acetyl-D-galactosamine.

3.1: Introduction.

N-Acetyl-D-galactosamine has an important role as a constituent of many N-glycoproteins and oligosaccharides. The N-glycoproteins are involved in many biological recognition processes including the control of blood group type, cell growth and differentiation, bacterial and viral infection and the uptake of macromolecular substances.[105,106] The sugar itself, also possesses interesting properties such as anti-tumour activity.[107]

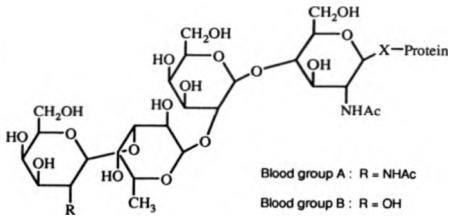
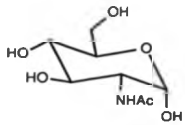


Fig. 3.1: The structure of the blood group antigen.

N-acetyl-D-galactosamine is very expensive to purchase, making its synthesis of interest. N-Acetyl glucosamine is by contrast much cheaper (by a factor of over 100) and N-acetyl-D-glucosamine is, therefore, a potential starting material for the

synthesis of N-acetyl-D-galactosamine. The major source of N-acetyl-D-glucosamine is its extraction from chitin, a polymeric form of the sugar. The two structures are identical apart from the configuration of the groups attached to carbon atom C-4 in the sugar ring. To convert N-acetyl-D-glucosamine into N-acetyl-D-galactosamine requires that the configuration at the C-4 position be inverted without any of the other positions being affected.



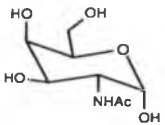


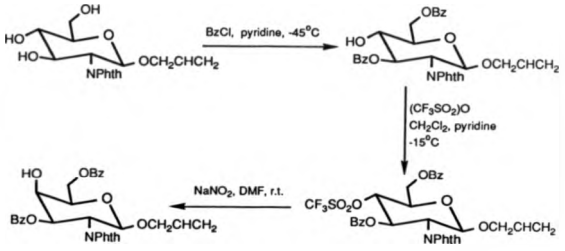
Fig. 3.2: N-Acetyl glucosamine
2-acetamido-2-deoxy-glucopyranose

N-Acetyl galactosamine
2-acetamido-2-deoxy-galactopyranose

The best way of achieving this is by specifically protecting all the other hydroxyl groups, or alternatively, deprotecting just the C-4 position. This allows reactions to be carried out only at the required centre. A particularly suitable means of achieving this protection is to utilise the ability of enzymes to deacetylate selectively certain positions in a peracetylated sugar. This technique provides a means of synthesing the required intermediate with the use of fairly mild reaction conditions.

It has already been noted in Chapter One that the selective deacetylation of monosaccharides can been carried out. This technique combined with an acyl group migration would provide a synthetic route to the required sugar specifically

deprotected at the C-4 position. The migration required is that from C-4 to C-6, which, as mentioned earlier is particularly favourable and can be achieved with a high conversion under the right conditions. This particular migration process is also utilised in one of the synthetic routes to sucralose, in this case the migration is from C-4 to C-6 in the pyranose ring of a sucrose derivative (2,3,3',4,4'-penta-*O*-acetyl sucrose converted to 2,3,3',4',6-penta-*O*-acetyl sucrose). This is a particularly interesting technique, as acyl migrations are usually a nuisance for the synthetic chemist, complicating reactions by forming unwanted products.



Scheme 3.1: An inversion of configuration by the displacement of a trifluoromethanesulphonyl (triflate) group.

A similar reaction to give inversion of configuration at the C-4 position of a glucosamine derivative by displacement of a triflate has recently been reported[108] (Scheme 3.1). The starting

material was however not so readily available requiring different protection of the C-1 position and the amine to achieve a good yield. The inversion of configuration has also been achieved using UDP-galactose-4-epimerase to generate uridine-5'-diphosphate galactose from the glucose derivative in yields of approximately 30%.[105]

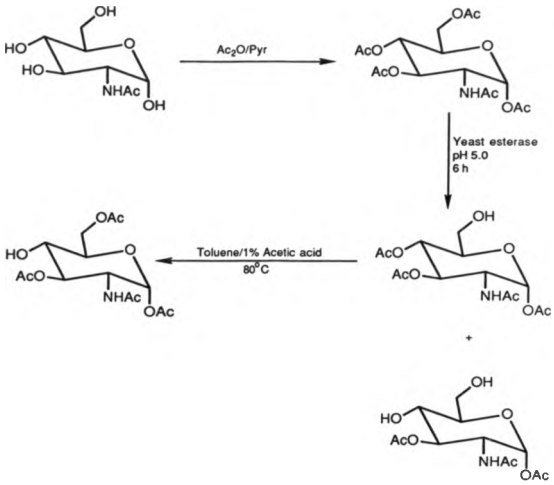
3.2: The synthesis of the protected intermediate.

The starting material for the synthesis was N-acetyl glucosamine (2-acetamido-2-deoxy-α-D-glucopyranose) which is commercially available at low cost. The first step in the synthesis was the peracetylation of the starting material. This was achieved by the standard method of treatment with an excess of acetic anhydride in pyridine, producing 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl glucosamine in 94% yield.[109](Scheme 3.2)

The selective deprotection of the primary position was the next step. Deacetylation of the C-6 acetyl group was achieved using an enzyme catalysed reaction. The reaction was catalysed by yeast esterase (Glaxo), a fairly crude enzyme preparation, in a phosphate/citrate buffer (pH 5.0) at 30°C. After six hours, t.l.c. indicated the complete hydrolysis of the peracetylated sugar. Two new products were indicated with one of these being very minor (Scheme 3.2).

After extraction, the products were purified by recrystallisation and then by flash chromatography of the residue. The products were then identified by 220MHz ^{1}H n.m.r. spectroscopy. The identification is readily made by comparison

with the fully acetylated derivative due to the effect of removing an acetate group.[110] The signal due to the proton attached to the position of deacylation moves upfield by up to 1.5 ppm due to the loss of the deshielding effect of the acetate group. The other proton signals remain in approximately the same position. As all the protons can be readily identified due to their different coupling constants and multiplicities the position of deacetylation may be deduced. The major product was the required partially protected sugar, 2-acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-α-D-glucopyranose.[111,112] The minor product was identified as 2-acetamido-2-deoxy-1,3-di-*O*-acetyl-α-D-glucopyranose which was obviously produced by a deacetylation of the major product. It would have been possible to stop the deacetylation reaction earlier and obtain less of this product but there would have been some starting material present. For reasons to be discussed later it was preferable to stop the hydrolysis after all the peracetate derivative had been deacetylated.



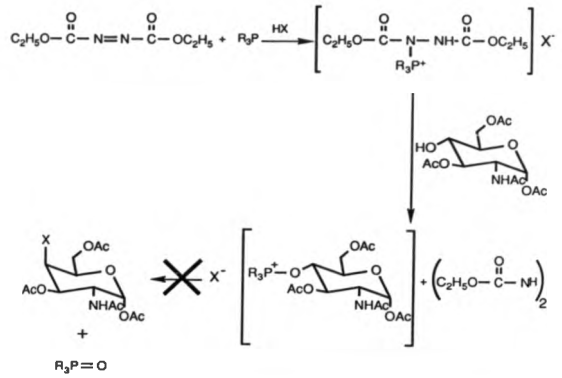
Scheme 3.2: The synthesis of the intermediate deprotected at the C-4 position.

An acyl migration was then utilised to produce the derivative with the required level and position of protection. As stated previously, the C-4 to C-6 migration is particularly facile. There is, however, an equilibrium due to the reversibility of the migration process in aqueous solution. This can be affected by changing the solvent to give a very good yield of the migration product.[39]

The 2-acetamido-2-deoxy-1,3,4-tri-*O*-acetyl glucopyranose was added to toluene in which it dissolved on heating to 80°C. Glacial acetic acid (1% v/v) was then added to the stirred solution. After 24 hours the presence of the required product was identified by 220MHz n.m.r. spectroscopy of the crude mixture. 2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose was recovered after purification in a yield of 80%. Identification by ^{1}H n.m.r. spectroscopy was again easy owing to the upfield shift of the H-4 signal and the downfield shift of the H-6 signal due to the effect of the acetyl group.

3.3: Inversion of configuration at C-4

Having synthesised the glucosamine derivative with the required protection, the next step in the synthesis was to invert the configuration at the C-4 centre. The initial choice for this reaction was to use the Mitsonobu reaction.[113] This would involve displacement of a phosphonium intermediate by a nucleophile such as the benzoate ion.[114] This displacement would proceed with the required inversion of configuration at C-4. Deprotection of the sugar would then yield the required product. Unfortunately when this displacement was attempted there was no reaction (Scheme 3.3).



Scheme 3.3: The Mitsonobu inversion reaction.

This, apparently, is due to the steric hindrance of the acetate groups around the molecule preventing the nucleophile from causing displacement of the leaving group as has been previously observed in a similar compound.[115]

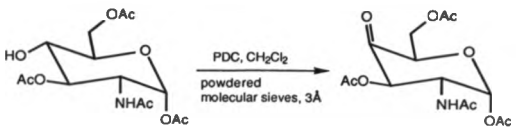
Another possibility for the inversion of the C-4 position was to use a two-step process of an oxidation step followed by a reduction step. The deprotected C-4 hydroxyl group would be oxidised to give the keto-derivative. Reduction of this with a suitable reducing agent should produce the required galacto-product. A series of reducing agents are available so the formation of the required configuration should be possible if it

was not formed by straightforward reduction with sodium borohydride.

There are several reported methods for the oxidation of sugar hydroxyl groups. These include using ruthenium tetraoxide, Swern oxidations, pyridinium chlorochromate and pyridinium dichromate.[116,117] These are all relatively mild methods for oxidation.

It was decided to use pyridinium dichromate to oxidise the glucosamine derivative. It has been reported that this reaction is greatly improved by the addition of powdered molecular sieves[118](Scheme 3.4). These were therefore used.

After 24 hours, t.l.c. on alumina showed the presence of one major product. After a purification step the ^1H n.m.r. spectrum identified the product as the required keto sugar. This identification was made due to the signal corresponding to H-3 collapsing from a triplet to a doublet, indicating that one of the couplings has disappeared. The signal due to H-4 was no longer present in the spectrum as would be expected for the keto-sugar. The signal due to H-2 had not noticeably changed in multiplicity and that due to H-5 was partially overlapping. The reaction was not completely clean so attempts were made to purify the keto-sugar by chromatography. The product, however, decomposed rapidly to give several products. This decomposition appeared to occur particularly during purification by chromatography on silica gel. However, decomposition also occurred during other means of purification.

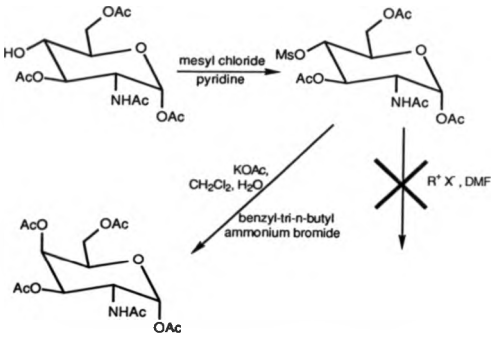


Scheme 3.4: The oxidation of 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose.

A reduction reaction was attempted on a crude sample of the keto derivative using sodium borohydride. This led to a mixture of products with no one compound appearing to be prevalent. This again may be due to the instability of the sugar. The instability of the keto-sugar suggested that an alternative method would be preferable.

An alternative method is to convert the unprotected hydroxyl group to a more reactive leaving group. The 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose was converted to the 4-*O*-mesyl derivative. This was achieved by the addition of a slight excess of mesyl chloride to a stirred solution of the 4-OH derivative in pyridine at 0°C (Scheme 3.5). The mesyl derivative was produced in good yield. Displacement of this group was not, however, found to be possible. This was a little surprising as similar displacements have been successful on sugar derivatives.[119,120] The attempted displacement by acetate or benzoate ions from the sodium salts in dimethylformamide or acetonitrile did not produce any reaction at room temperature or on gentle heating (Scheme 3.5). Refluxing the mixture just caused the decomposition of the

glucosamine derivative. The same was also found to be true when the tosylate derivative, synthesised by the treatment of the protected sugar with tosyl chloride, was used instead. A milder approach to this displacement was to use a phase transfer catalyst for the reaction. There have been many reports of this type of catalysis being successfully employed in nucleophilic displacement reactions.[118]



Scheme 3.5: The synthesis and displacement of a methanesulphonyl group.

A phase transfer catalysis reaction was attempted using benzyl-tri-n-butyl ammonium bromide as the transfer catalyst. The 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-*O*-mesyl-α-D-

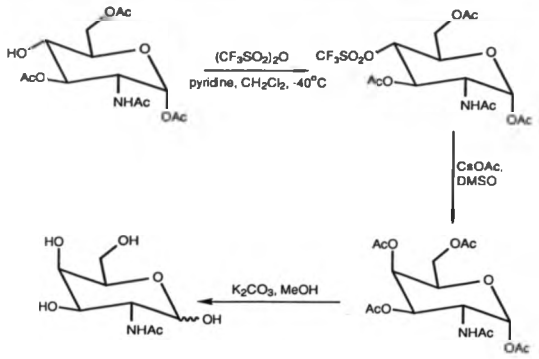
glucopyranose was dissolved in dichloromethane. To this, was added, a solution of potassium acetate in water and a small quantity of the catalyst (Scheme 3.5). T.l.c. indicated the presence of a small amount of a compound with a higher Rf value than the mesylate. After separation and purification of this compound analysis by ^1H n.m.r. spectroscopy showed that there had been an inversion of the configuration at C-4 to give the galacto- derivative. The ^1H n.m.r. spectrum of the product showed substantial changes to the signals of H-3, H-4 and H-5. Compared to the peracetylated *gluco*-derivative the signals appeared at similar chemical shifts. There were, however, substantial changes in the coupling constants and in the splitting patterns of each of the signals. This allowed the sugar to be identified as the *galacto*-derivative.

The yield for this inversion step was, however, very poor. Despite the presence of the mesyl leaving group, very little displacement was actually occurring. Mesylate and tosylate are very good leaving groups compared to carboxylic esters. The triflate group is however approximately 40000 times more reactive towards displacement.[121] Triflates have previously been used as effective leaving groups in sugars.[122,123]

The triflate of N-acetyl-1,3,6-tri-*O*-acetyl-α-D-glucopyranose was synthesised by treatment of the sugar with triflic anhydride in pyridine/dichloromethane at -40°C (Scheme 3.6). The reaction was followed by t.l.c. and gave one product. The derivative was found to be relatively stable despite the reactivity of the triflate and could be purified to some degree. In general as little as possible was done with the derivative before further reaction so as to avoid any decomposition. The

most convenient displacement was by an acetate ion forming the peracetylated D-galactosamine with all the hydroxyl groups protected with the same group. To achieve the required displacement the nucleophilic acetate ions need to be in the form of a loose ion pair. This requires as large a cation as possible for the acetate salt. Caesium acetate is the best available salt for this displacement and was formed by the treatment of caesium carbonate with acetic acid followed by evaporation of the solvents under reduced pressure.

The reaction is likely to be very solvent dependent due to the need to solvate the nucleophilic acetate ions. An aprotic solvent such as acetonitrile, dimethylformamide or dimethyl sulphoxide is necessary for this displacement reaction. Using acetonitrile as the solvent for the reaction successfully produced the required galactosamine derivative. The yield was again unsatisfactorily low possibly due to the solubility of the caesium acetate not being high enough.

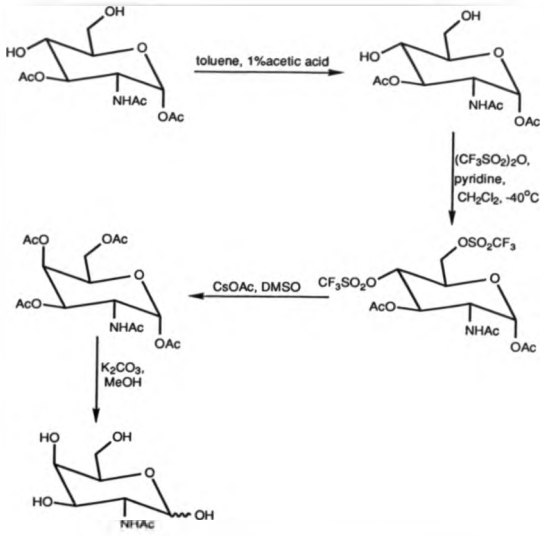


Scheme 3.6: The inversion of configuration by displacement of a triflate group by an acetate group.

Changing the solvent to dimethyl sulphoxide increased the solubility of the salt so that the required amount of salt was all in solution. When a solution of 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-*O*-trifluoromethanesulfonyl-α-D-glucosamine in dimethyl sulphoxide was stirred with caesium acetate for 12 hours, t.l.c. showed one major product of high Rf and two minor products with lower Rf. The solvent was evaporated under reduced pressure and the residue peracetylated using acetic anhydride in pyridine. T.l.c. now showed the presence of only one compound, corresponding to the compound of high Rf. Analysis by 220MHz n.m.r. identified this product as 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose. Recrystallisation gave the product in 81% yield, based on the

derivative deprotected at C-4. This yield was a considerable improvement on any of the other methods of inversion. Deacetylation of the fully acetylated sugar was achieved using potassium carbonate in methanol at -10°C for 1 hour to yield 2-acetamido-2-deoxygalactopyranose in 93% yield (Scheme 3.6).

The minor product from the enzymatic step was identified as 2-acetamido-2-deoxy-1,3-di-*O*-acetyl-α-D-glucopyranose. The production of a small quantity of a diacetate is to be expected due to further deacylation of the first product. This is not a major inconvenience due to the reactions used thereafter. The required migration step of the reaction will obviously not occur and it is unlikely that any of the much less favoured migrations will. The addition of triflic anhydride will form the required C-4 triflate together with the one at C-6. Displacement with caesium acetate in dimethylsulphoxide will simply displace the triflate at C-6. The displacement by acetate at C-4 will give the required inversion of configuration as seen for the mono-deprotected derivative. The product of this displacement is the same as before and deprotection will give 2-acetamido-2-deoxy-D-galactopyranose (Scheme 3.7). This means that purification of the reaction mixture to remove the only byproduct formed in any quantity is not necessary.



Scheme 3.7: The reaction of the minor product, 2-acetamido-2-deoxy-1,3-di-*O*-acetyl-α-D-glucopyranose, following the same synthetic pathway.

All of the above reactions are performed on a multi-gram scale with high yields. The overall yield for the synthesis is calculated to be 51% based on the yields for each step. This figure would have been slightly higher if the minor product described above had not been removed. This compares

favourably with previous methods for the inversion of configuration at the C-4 position. The recent publication by El Sokkary *et al* achieved a 63% yield for the inversion beginning with and finishing with a protected derivative rather than starting with a readily available sugar and other methods have only achieved poor yields for the inversion step.[108,120]

This synthetic route is interesting in that it contains a selective enzymatic deacetylation, the use of an acyl migration as a synthetic step and the displacement of a triflate group by an acetate group with the inversion of configuration at a sugar ring carbon centre. The use of partially protected sugars produced by enzymatic deacetylation as synthetic intermediates for further reaction is demonstrated in this conversion.

Chapter Four: Experimental Details.

4.1: General.

Chemicals were either purified using literature methods or purchased as the highest available grade.[124] The wheat germ lipase was obtained from the Sigma Chemical Company Ltd. The yeast esterase was supplied in small quantity by Tate and Lyle who received it from Glaxo Pharmaceutical Company. The lipase from *Aspergillus niger* was obtained from the Amano Pharmaceutical Company.

The n.m.r. spectra of the crude products were obtained using a Perkin Elmer R34 spectrometer operating at 220 MHz. Chemical shifts are reported in ppm relative to tetramethylsilane (0.00 ppm). N.m.r spectra of pure products were obtained using a Bruker WH 400 spectrometer. Long range ^{13}C-^{1}H shift correlation was carried out by a heteronuclear COSY-type experiment, with the use of a composite 180° carbon pulse, and refocussing delays of 3.7 and 1.85 ms.

The multiplicities of the ^{1}H n.m.r. signals, where applicable, are abbreviated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet.

Mass spectra were obtained using a Kratos MS-80 spectrometer. In general spectra were recorded using CI with ammonia as the carrier gas. The FAB spectra were recorded using diethanolamine and p-nitrobenzyl alcohol as matrices.

Microanalyses were carried out by Medac Ltd. Melting points are uncorrected.

Unless otherwise stated t.l.c. analyses were run on glass plates coated with silica gel (Merck 60_{F254}, 0.25mm). The glucose pentaacetate deacetylation products were eluted with petroleum ether (b.p. 40-60°C): ethyl acetate (1:9) and the sucrose octaacetate deacetylation products with both petroleum ether (b.p. 40-60°C): ethyl acetate (1:9) and ethyl acetate: ethanol: water (45:5:1) for the lower acetate classes. The compounds in the synthesis of N-acetyl galactosamine were eluted using ethyl acetate: ethanol: water (45:5:1) unless otherwise stated. Visualisation was achieved where possible by U.V. fluoresence quenching. For most of the analyses it was necessary to spray the plates with 10% H_2SO_4 in ethanol followed by heating to observe the sugars.

Preparative t.l.c. purifications were run on glass backed plates coated with silica gel (Merck 60_{F254}, 1.0 mm, 20cmx20cm).

4.2: The hydrolysis of sucrose octaacetate catalysed by yeast esterase.

4.2.1: The hydrolysis of a suspension of sucrose octaacetate catalysed by yeast esterase.

A suspension of sucrose octaacetate (100mg, 0.14mmol) in acetate buffer (0.2M, pH 4.8, 10.0ml) was stirred at room temperature with yeast esterase (50mg). The reaction was stopped after intervals of 6 h, 18 h and 30 h by freezing. After drying the mixture by lyophilisation, the mixture of products was perdeuterioacetylated by treatment with d_6-acetic

anhydride (0.4ml, 4.9mmol) in pyridine (0.4ml) at 50°C for 4h. The solvents were removed under reduced pressure. The products were then analysed by mass spectroscopy. All samples were analysed in duplicate to ensure the validity of the data.

The method for the assignment of the acetyl signals was provided initially by Tate and Lyle from the studies published by Rathbone.[83] We have since confirmed these by a 2-D n.m.r. experiment as described below.

4.2.2: The hydrolysis of a solution of sucrose octaacetate using yeast esterase.

Sucrose octaacetate (100mg) was stirred in acetate buffer (0.2M, pH 4.8, 120ml) at room temperature. After a period of 30 minutes the solution was filtered to remove the excess sucrose octaacetate. Yeast esterase (50mg) was added to the solution and aliquots were removed at various time intervals. The treatment of the aliquots was as described above.

4.3: The Deacetylation of Glucose Derivatives.

4.3.1: α- and β-methyl-D-glucopyranoside tetraacetates.

To a solution of methyl-α-D-glucopyranoside (5.0g, 0.026M) in pyridine (10ml) was added acetic anhydride (20ml, 0.21mol). The reaction mixture was stirred overnight at room temperature. On completion of the reaction dilute HCl (50ml) was added and the solution was extracted using diethyl ether (3 x 50ml). The combined ethereal extracts were dried over

anhydrous magnesium sulphate and evaporated to dryness under reduced pressure to give a syrup. This was crystallised from ethanol to yield large colourless crystals of the tetraacetate (6.02g, 64%) m.p. 104°C, δ_H (220MHz; d_5-pyridine) 5.95 (1H, t, J 9.8Hz, 3-H), 5.47 (1H, t, J 9.8Hz, 4-H), 5.30 (1H, dd, J 10.3, 3.7, 2-H), 5.20 (1H, d, J 3.7Hz, 1-H), 4.48 (1H, dd, J 4.9, 12.3Hz, 6_a-H), 4.35 (1H, dd, J 1.9, 12.3Hz 6_b-H), 4.13 (1H, ddd(m), 5-H), 3.45 (3H, s, O-Me), 2.02 (9H, s), 1.95 (3H, s). Lit. m.p. 105°C.[125]

The methyl-β-D-glucopyranoside (5.00g, 0.026mol) was peracetylated in identical fashion to yield white crystals. (9.16g, 96% yield) m.p. 105°C, δ_H (220MHz; $CDCl_3$) 5.24 (1H, t, J 9.8Hz, 3-H), 5.14 (1H, t, J 9.8Hz, 4-H), 5.02 (1H, broad t, J 9.5Hz, 2-H), 4.47 (1H, d, J 8.3Hz, 1-H), 4.32 (1H, dd, J 4.9, 12.5Hz, 6_a-H), 4.18 (1H, dd, J 12.5, 12.5, 6_b-H), 3.78 (1H, ddd(m), 5-H), 3.55 (3H, s, O-Me), 2.12 (3H, s), 2.08 (3H, s), 2.05 (3H, s), 2.02 (3H, s). Lit. m.p. 104-105°C.[125]

4.3.2: The enzymatic hydrolysis of methyl-α-D-glucopyranoside tetraacetate catalysed by *A. niger* lipase.

A suspension of methyl-α-D-glucopyranoside tetraacetate (50mg, 0.14mmol) in phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7.9, 1.28ml, 0.5M) was stirred at 30°C with Lipase A from *A. niger* (64mg). The reaction was stopped at intervals of up to 30h. The reactions were immediately frozen and lyophilised. The residue was then treated with d_6-acetic anhydride (200µl, 2.1mmol) in pyridine (1.5ml) and left for 12h. Analysis of the

product was carried out by ^1H n.m.r. and mass spectroscopy. The ^1H n.m.r. spectrum showed that three products, α- and β-D-glucose pentaacetate and the starting material, methyl-α-D-glucopyranoside tetraacetate were present in the perdeuterioacetylated sample, indicating that deprotection of the anomeric C-1 position had occurred.

4.3.3: Control experiments carried out on the hydrolysis of methyl-D-glucopyranoside.

4.3.3a: A study on the effect of the enzyme.

A suspension of methyl-α-D-glucopyranoside tetraacetate (50mg, 0.14mmol) in phosphate buffer (pH 7.9, 1.28ml, 0.5M) was stirred in the absence of enzyme at 30°C for 18h. The reaction was lyophilised and the residue treated with d_6-acetic anhydride (0.5ml, 5.3mmol) in pyridine (1ml). The analysis was carried out as described above. The ^1H n.m.r. spectrum showed that the only product present was the starting material, indicating that no deprotection of the C-1 position had occurred.

4.3.3b: A study of the effect of the enzyme on the free sugar.

A suspension of methyl-α-D-glucopyranoside (50mg, 0.14mmol) and *A. niger* lipase A (64mg) in phosphate buffer (pH 7.9, 1.28ml, 0.5M) was stirred at 30°C for 18h. The reaction was lyophilised and the residue treated with acetic anhydride (0.5ml, 5.3mmol) in pyridine (1ml). The analysis was carried out as described above. The ^1H n.m.r. spectrum showed that

three products, the starting material, methyl-α-D-glucopyranoside tetraacetate and small amounts of α- and β-D-glucose pentaacetate were present in the perdeuterioacetylated sample, indicating that deprotection of the anomeric C-1 position had again occurred.

4.3.3c: A study of the other anomer of methyl-D-glucopyranoside.

A suspension of methyl-β-D-glucopyranoside tetraacetate (50mg, 0.14mmol) and *A. niger* lipase A (64mg) in phosphate buffer (pH 7.9, 1.28ml, 0.5M) was stirred at 30°C for 18h. The reaction was lyophilised and the residue treated with d_6-acetic anhydride (0.5ml, 5.3mmol) in pyridine (1ml). The analysis was carried out as described above. The ^1H n.m.r. spectrum showed that three products, α- and β-D-glucose pentaacetate and the starting material, methyl-β-D-glucopyranoside tetraacetate were present in the perdeuterioacetylated sample, indicating that deprotection of the anomeric C-1 position had once more occurred.

4.3.4: The hydrolysis of β-D-glucose pentaacetate.

A suspension of β-D-glucose pentaacetate (250mg, 0.64mmol) and lipase A from *A. niger* (320mg) in phosphate buffer (pH 7.9, 6.4ml, 0.5M) was stirred at 30°C. The reaction was stopped at intervals between 40 and 150 minutes. The

solution was lyophilised and a small sample (10mg) removed. The remainder was treated as below.

4.3.4a: Separation of partly acetylated products.

After lyophilisation the residue was dissolved in ethyl acetate:ethanol (1:1, 25ml). Silica (1g, silica gel 60, particle size 0.040-0.063mm) was added. The solvent was evaporated under reduced pressure and the silica gel was packed on top of a column of silica gel 60, (25mm diam., 60g). The column was eluted with the following solvents: petroleum (b.p. 40-60°C):ethyl acetate (1:1, 250ml; 4:6, 125ml; 3:7, 250ml; 2:8, 125ml; 1:9 125 ml); ethyl acetate (250ml); ethyl acetate:ethanol (95:5, 125ml; 9:1,125ml; 8:2, 125 ml). Fractions (20ml) were collected and analysed by t.l.c., solvent system petroleum ether (b.p. 40-60°C):ethyl acetate (1:9). If cross-contamination by another class of partial hydrolysis products was observed, the fraction was further separated by preparative t.l.c. The solvent was evaporated under reduced pressure and the residue was perdeuterioacetylated using d_6-acetic anhydride (0.2ml, 2.1mmol) in pyridine (0.5ml) over 12h. The solution was evaporated under reduced pressure and the residue was examined by ^1H n.m.r. and mass spectroscopy. A sample of the total mixture of hydrolysis products removed prior to the separation, was also perdeuterioacetylated and analysed in the same way.

4.4: The deacetylation of sucrose octaacetate catalysed by wheat germ lipase.

4.4.1: Small scale hydrolysis of sucrose octaacetate catalysed by wheat germ lipase.

A suspension of sucrose octaacetate (58mg, 0.09mmol) in phosphate buffer (pH 7.0, 0.5M, 0.8ml) with wheat germ lipase (40mg) was stirred at 30°C. The reaction was stopped at various times from 2 to 102h.

The reaction mixture was lyophilised and then perdeuterioacetylated using d_6-acetic anhydride (0.2ml, 2.1mmol) in pyridine (1ml). The solvent was evaporated under reduced pressure and the residue analysed by ^1H n.m.r. and mass spectroscopy.

4.4.2: Large scale hydrolysis of sucrose octaacetate catalysed by wheat germ lipase.

A suspension of sucrose octaacetate (700mg, 1.03mmol) in phosphate buffer (KH_2PO_4, Na_2HPO_4, 100ml, pH 7.0, 0.5M), was stirred at 30°C with wheat germ lipase (500mg). The reaction was stopped at various intervals from 12 to 48h by immediate freezing. After drying by lyophilisation a small sample was removed for determination of the composition of the mixture.

The products of the reaction were extracted using a combination of ethyl acetate, ethanol and water (45:5:1) and filtered to remove the insoluble enzyme. The remainder was adsorbed on to silica gel and then separated by column

chromatography on silica gel (80g, 25mm diameter). The column was eluted with the following solvents: light petroleum (b.p. 40-60°C):ethyl acetate (1:4, 250ml; 1:7, 250ml; 1:19, 250ml); ethyl acetate (250ml); ethyl acetate:ethanol (99:1, 250ml; 95:5, 250ml; 9:1, 500ml). This is the general procedure, minor variations were made according to the content of the mixture.

Fractions (20ml) were collected, analysed by t.l.c. and collected into classes. If contamination of a class was observed the fraction was further separated by preparative t.l.c. The solvent was evaporated under reduced pressure and the residue was perdeuterioacetylated using d_6-acetic anhydride (0.2ml, 2.1mmol) in pyridine (0.5ml) over 12h. The solution was evaporated under reduced pressure and the residue examined by ^1H n.m.r. and mass spectroscopy. A small sample of the crude mixture was also perdeuterioacetylated and analysed by the same method.

4.5: The Synthesis of 2-Acetamido-2-deoxy-α-D-galactopyranose.

2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose.

2-Acetamido-2-deoxy-α-D-glucopyranose (11g, 50mmol) was treated with acetic anhydride (47ml, 0.50mol) in pyridine (50ml) at room temperature. After 12h, t.l.c. analysis showed the formation of one product (Rf=0.8, ethyl

acetate:ethanol:water 45:5:1). The mixture was evaporated under reduced pressure to give a syrup which was crystallised from ethanol to give 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (17.73g, 92%) m.p. 136-137°C; $[\alpha]_D$ +91° (c 0.6, MeOH); δ_H (400MHz; $CDCl_3$) 6.13 (1H, d, J 3.6Hz, 1-H), 5.63 (1H, d, J 9.0Hz, N-H), 5.19 (2H, m, 3-H 4-H), 4.45 (1H,ddd, J 3.6, 9.1, 10.5Hz, 2-H), 4.21 (1H, dd, J 4.0,12.5Hz, 6_a-H), 4.03 (1H, dd, J 2.4,12.5Hz, 6_b-H), 3.96 (1H, ddd, J2.4, 4.0, 9.7Hz, 5-H), 2.16 (3H, s), 2.05 (3H, s), 2.02 (3H, s), 2.01 (3H, s), 1.90 (3H, s); ^{13}C: 171.5, 170.5, 169.7, 168.9, 168.4, 90.6, 70.6, 69.6, 67.5,61.4, 51.0, 22.8, 20.7, 20.5, 20.4; m/z 390 (MH^+), 330, 289, 270, 228, 210, 168. Lit. m.p. 134-135°C [126], 137°C [127], 135-137°C [128], 139.5-140.5°C [129], $[\alpha]_D$ +87.4° (c 1.03, $CHCl_3$) [107], +92° (c 1.0, $CHCl_3$) [127,109], +94° (c 1.0, $CHCl_3$) [126].

2-Acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-α-D-glucopyranoside.

2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (33.15g, 85mmol) was suspended in citrate/phosphate buffer (350ml, pH 5.0, 50mmol/100mmol) at 30°C. Yeast esterase (1.003g) was added and the mixture stirred for 24h. The solution was evaporated under reduced pressure and the residue was extracted with ethyl acetate (2x100ml) and ethanol (2x100ml). The combined extracts were filtered and evaporated under reduced pressure to give a syrup, which was identified as 2-acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-α-D-glucopyranose (Rf=0.56, ethyl acetate: ethanol:water 45:5:1). A minor product (Rf=0.33) present was identified by ^1H n.m.r. as

2-acetamido-2-deoxy-1,3-di-*O*-acetyl-α-D-glucopyranose. The mixture was used without further purification (see chapter 3).

This reaction was repeated on a smaller scale using 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (8.005g, 20mmol). On this scale the compound was selectively hydrolysed to give, after crystallisation from ethanol, 2-acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-α-D-glucopyranose (5.74g, 80%) m.p. 110°C (from ethanol); $[\alpha]_D$ +57° (c 1.0, $CHCl_3$); (Found: C, 47.6; H, 6.2; N, 4.0. $C_{14}H_{21}NO_{10}$ requires C, 48.4; H, 6.1; N, 4.0%); δ_H (400MHz; $CDCl_3$) 6.15 (1H, d, J 3.6Hz, 1-H), 5.76 (1H, d, J 9.0Hz, N-H), 5.25 (1H, dd, J 9.6, 10.8Hz, 3-H), 5.14 (1H, dd, J 9.7, 9.7Hz, 4-H), 4.43 (1H, ddd, J 3.6, 9.0, 10.9Hz, 2-H), 3.78 (1H, ddd, J 2.2, 4.3, 10.1, 5-H), 3.66 (1H, dd, J 2.2, 12.8Hz, 6_a-H), 3.55 (1H, dd, J 4.4, 12.8, 6_b-H), 2.16 (3H, s), 2.05 (3H, s), 2.04 (3H, s), 1.91 (3H, s); ^{13}C: 171.7, 170.0, 169.7, 168.7, 90.6, 72.0, 70.5, 67.8, 60.8, 51.1, 22.8, 20.7, 20.6, 20.4; m/z FAB -EI (NBA), 453 $(M+NBA)^+$, 411, 348, 288.

2-Acetamido-2-deoxy-1,3-di-*O*-acetyl-α-D-glucopyranose

This is the minor product from the previous reaction formed by the further deacetylation of the required product.

δ_H (400MHz; $CDCl_3$) 6.11 (1H, d, J 3.6Hz, 1-H) 5.71 (1H, d, J 9.0Hz, N-H), 5.12 (1H, dd, J 9.2, 11.0Hz, 3-H), 4.3 (1H, ddd, J 3.7, 9.0, 11.1Hz, 2-H), 3.90 (1H, dd, J 9.2, 9.8Hz, 4-H), 3.84(2H, d, J 3.5Hz, 6_a-H & 6_b-H), 3.7 (1H, dt, J 9.8, 3.5Hz, 5-H), 2.17 (3H, s), 2.13 (3H, s), 1.93 (3H, s).

2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranoside.

2-Acetamido-2-deoxy-1,3,4-tri-*O*-acetyl-α-D-glucopyranose (16.046g, 46mmol) was added to toluene (300ml) and heated to 80°C. Glacial acetic acid (3ml) was added to the solution which was heated at 80°C for 24h. The solvent was removed under reduced pressure to give a brown syrup which was identified as the required product by ^1H n.m.r. spectroscopy. The syrup was dissolved in ethyl acetate and filtered through silica. The solvent was removed *in vacuo* and the residue crystallised from dichloromethane to yield 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose (14.104g, 87.9%), m.p. 160-161°C ; $[\alpha]_D$ +62° (c 1.0, $CHCl_3$); (Found: C, 48.28; H, 6.12; N, 4.03. $C_{14}H_{21}NO_{10}$ requires C, 48.41; H, 6.09: N, 4.03%); δ_H (400MHz; $CDCl_3$) 6.12 (1H, d, J 3.7Hz, 1-H), 5.92 (1H, d, J 8.9Hz, N-H), 5.08 (1H, dd, J 9.2., 11.0Hz, 3-H), 4.50 (1H, dd, J 3.6, 12.4Hz, 6_a-H), 4.31 (1H, ddd, J 3.6, 8.9, 11.0Hz, 2-H), 4.18 (1H, dd, J 2.2, 12.4Hz, 6_b-H), 3.82 (1H, ddd, J 9.5, 2.2, 3.6Hz, 5-H), 3.6 (1H, dd, J 9.2, 9.6Hz, 4-H), 2.15 (3H, s), 2.10 (3H, s), 2.09 (3H, s) 1.90 (3H, s); ^{13}C: 172.0, 171.7, 170.3, 169.0, 90.8, 72.6, 72.0, 67.8, 62.4, 51.1, 22.8 (2C), 20.8, 20.6; m/z 370 $(MH.Na)^+$, 348 (MH^+), 329, 307, 288, 228, 186, 176, 168.

2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-trifluoromethanesulfonyl glucopyranose.

2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose (4.057g, 11.7mmol) was dissolved in dichloromethane (40ml) and pyridine (4ml) under an

atmosphere of nitrogen. The solution was cooled to -40°C using a dry ice/acetonitrile bath. Triflic anhydride (2.5ml, 14.9mmol) was added slowly to the solution with stirring.

The reaction was followed by t.l.c. (ethyl acetate /ethanol/water; 45:5:1). After 4h there was one product (Rf 0.77) which was identified as the required product by n.m.r. spectroscopy. On completion of the reaction, dichloromethane (60ml) was added together with water/ice (100ml). Sodium carbonate (0.2g) was added to the mixture. The dichloromethane layer was removed and washed with dilute HCl (1x100ml) and then saturated NaCl solution(1x100ml). The dichloromethane was removed under reduced pressure to yield 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-trifluoromethanesulfonyl glucopyranose as a syrup/glass (5.171g, 95%) δ_H (220MHz; $CDCl_3$) 6.68 (1H, d, J 9.8Hz, N-H), 6.21 (1H, d, J 3.7Hz, 1-H), 5.51 (1H, t, J 9.8Hz, 3-H), 5.23 (1H, t, 9.8Hz, 4-H), 4.64 (1H, dt, J 3.7, 9.8Hz, 2-H), 4.37 (1H, dd, J 3.6, 12.4Hz, 6_a-H), 4.25 (2H, m, 5-H 6_b-H), 2.18 (3H, s), 2.16 (3H, s), 2.14 (3H, s), 2.00 (3H, s); m/z 467 (MH^+), 449, 425, 407.

<u>2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl galactopyranose.</u>

2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-trifluoromethanesulfonyl glucopyranose (4.961g, 10.6mmol) was dissolved in DMSO (40ml) under an atmosphere of nitrogen. Caesium acetate (4.0g, 20.8mmol) was added and the mixture was stirred at room temperature for 14h. The DMSO was removed by distillation under reduced pressure. T.l.c. showed

that one major product had been produced (Rf 0.62) together with some minor products with lower Rf. The syrup was dissolved in pyridine (10ml), acetic anhydride (10ml, 0.11mol) was added and the solution was stirred at room temperature for 6 h. T.l.c. showed only one product (Rf 0.62).

Ice/water (50ml) and saturated sodium chloride solution (50ml) were added, and the mixture was extracted with dichloromethane (3x100ml) and ethyl acetate (2x50ml). The organic fractions were combined, washed with dilute HCl (50ml) and evaporated to dryness under reduced pressure. The crude syrup was crystallised from ethanol (3.68g, 89%); m.p. 169-171°C; $[\alpha]_D$ +97° (c 1.0, $CHCl_3$); δ_H (400MHz; $CDCl_3$) 6.15 (1H, d, J 3.6Hz, 1-H), 5.82 (1H, d, J 9.0Hz, N-H), 5.36 (1H, dd, J 0.7, 3.1Hz, 4-H), 5.14 (1H, dd, J 3.2, 11.7Hz, 3-H), 4.63 (1H, ddd, J 3.6, 9.0, 11.6Hz, 2-H), 4.20 (1H, ddd, J 0.9, 6.6, 6.8Hz, 5-H), 4.04 (1H, dd, J 6.8, 11.2Hz, 6_a-H), 3.99 (1H, dd, J 6.6, 11.2Hz, 6_b-H), 2.10 (6H, s), 1.97 (3H, s), 1.96 (3H, s), 1.88 (3H, s); ^{13}C 170.75, 170.10, 170.04, 169.95, 168.67, 91.06, 68.33, 67.57, 67.45, 66.54, 61.10, 46.75, 22.77, 20.62, 20.43, 20.36; m/z 390 (MH^+), 330, 289, 279, 244, 226, 210. Lit. m.p. 178°C, $[\alpha]_D$ +102° (c 1.6, $CHCl_3$) [130].

2-Acetamido-2-deoxy-D-galactopyranose.

2-Acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl galactopyranose (1.17g, 3.0mmol) was dissolved in methanol (100ml) and cooled to -10°C. Anhydrous potassium carbonate (0.83g, 6.01mmol) was added and the mixture was stirred for 75min. T.l.c. (ethyl acetate/ethanol/water 45:5:1) showed one product (Rf 0.03). The mixture was dried, applied to a silica gel column (10g, 10mm diam.) and eluted with ethyl

acetate/ethanol (4:1). The product was a syrup (0.617g, 93%). A small amount was crystallised from dichloromethane/diethylether; m.p. 156-158°C; $[\alpha]_D$ +83° (c 0.2, H_2O); δ_H (400MHz; $CDCl_3$) α-anomer 5.13 (1H, d, J 3.7Hz, 1-H), 4.04 (1H, dd, J 3.7, 11.2Hz, 2-H), 4.01 (1H, t, J 6.2Hz, 5-H), 3.90 (1H, d, J 3.2Hz, 4-H), 3.83 (1H, dd, 3.2, 11.1Hz, 3-H), 3.65 (2H, d, J 6.2Hz, 6-H), 1.96 (3H, s), β-anomer 4.55 (1H, d, J 8.4Hz, 1-H), 3.84 (1H, d, J 3.2Hz, 4-H), 3.78 (1H, dd, J 8.4, 10.8Hz, 2-H), 3.67 (3H, 3-H, 6-H); ^{13}C α-anomer 175, 91, 71, 69, 67, 61, 51, 22.5, β-anomer 175, 96, 75, 71, 68, 61, 54, 22.8. Lit. m.p. 159-160°C, $[\alpha]_D$ +86° (c 1.0, H_2O) [131]. ^1H n.m.r. identical with a mixture of anomers of an authentic sample of the compound.

2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-keto glucopyranose.

2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl glucopyranose (0.694g, 2mmol) was dissolved in dichloromethane (25ml). Pyridinium dichromate (1.504g, 4mmol) was added together with powdered molecular sieves (0.5g) and the reaction mixture was stirred for 24h. The reaction was followed by t.l.c. (alumina plates eluted three times with ethyl acetate).

The reaction mixture was filtered through celite and eluted with dichloromethane. The solvent was removed under reduced pressure and the residue was applied to a silica gel column (35g) which was eluted with ethyl acetate (550ml).

The compound was found to partially decompose on the silica column. An n.m.r. spectrum of the compound indicated that the major product was the 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-keto glucopyranose. δ_H (220MHz; $CDCl_3$) 6.7 (1H, d, J

7.2Hz, N-H), 6.45 (1H, d, J 8.9Hz, 1-H), 5.65 (1H, d, J 12.2Hz, 3-H), 4.9 (1H, ddd, J 3.6, 8.5, 12.2Hz, 2-H), 4.65 (1H, dd, J 0.9, 4.9Hz, 5-H), 4.5 (1H, d, J 0.9, 12.3Hz, 6_a-H), 4.4 (1H, dd, J 4.9, 12.3Hz, 6_b-H), 2.25 (3H, s), 2.22 (3H, s), 2.1 (3H, s), 2.01 (3H, s).

Mitsonobu inversion reaction.

A solution of 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose (50mg, 0.14mmol), triphenylphosphine (113mg, 0.42mmol) and benzoic acid (52mg, 0.42mmol) in THF (0.5ml) was stirred at room temperature. A solution of diethyl azodicarboxylate (70µl, 0.42mmol) in THF (0.15ml) was added to the solution over a period of five minutes. The reaction was followed by t.l.c. Analysis by 220MHz n.m.r. indicated that the intermediate had formed but that no displacement had occurred.

2-Acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-4-methanesulphonyl glucopyranose.

A solution of 2-acetamido-2-deoxy-1,3,6-tri-*O*-acetyl-α-D-glucopyranose (50mg, 0.14mmol) was dissolved in pyridine (0.5ml) under an atmosphere of nitrogen. The solution was cooled to 0°C. Methanesulphonyl chloride (100µl, 1.3mmol) was added slowly to the solution with stirring. After purification by chromatography on silica gel (5g), eluting with petroleum ether (40-60°C)/ethanol (1:9) the derivative was dissolved in DMF and heated first to 80°C and then to reflux with sodium benzoate (40mg, 0.28mmol). At first, analysis of the reaction showed no displacement and after reflux, analysis indicated that the product had decomposed.

Appendix 1: The study of sucrose acetates by n.m.r. spectroscopy.

Sucrose octaacetate was analysed by n.m.r. spectroscopy in both d_6-dimethylsulphoxide and deuterium oxide. A heptaacetate, 2,3,6,1',3',4',6'-hepta-O-acetyl sucrose and a pentaacetate, 2,3,6,3',4'-penta-O-acetyl sucrose were also analysed using d_6-dimethylsulphoxide as solvent.

The sugar ring proton coupling constants were found to have extremely little variation in size. The only changes of any size observed were in the coupling constants between protons H-3', H-4' and H-5'. A variation of 1.4 Hz was observed in the $J_{3',4'}$ value of sucrose octaacetate when the spectrum was recorded in d_6-dimethylsulphoxide (6.6Hz) and in deuterium oxide (5.2Hz). However this variation only signifies a change of 7° in the torsion angle between the two protons, calculated using a modified Karplus equation. This shows that the structure of sucrose octaacetate is rigid in solution apart from slight flexing of the furanose ring. This is in agreement with the results found for sucrose in solution.[90] There is also, little apparent variation in the conformation of the structure when acetate groups are removed as the heptaacetate and pentaacetate have very similar coupling constants to the octaacetate.

Sucrose octaacetate

	d_6-DMSO	D_2O
a, b	J/Hz	J/Hz
1, 2	3.6	3.5
2, 3	10.4	10.3
3, 4	9.7	9.9
4, 5	9.7	9.9
3', 4'	6.6	5.2
4',5'	6.6	5.3

1',2,3,3',4',6,6'-hepta-O-acetyl sucrose

	d_6-DMSO
a, b	J/Hz
1, 2	3.6
2, 3	10.4
3, 4	9.5
4, 5	9.5
3', 4'	6.7
4', 5'	6.6

2,3,3',4',6-penta-O-acetyl sucrose

	d_6-DMSO
a, b	J/Hz
1, 2	3.6
2, 3	10.3
3, 4	9.4
4, 5	9.5
5, 6_a	1.8
5, 6_b	5.8
3', 4'	7.0
4', 5'	6.9

Appendix 2. Sucrose Acylations.

A considerable amount of work has been carried out on the acetylation of sucrose, although selective acetylation in high yield was not achieved. Most of this has been based around the acetylation work carried out by Tate & Lyle as described in Chapter 1. Their work used lipase P from *Pseudomonas* sp. to catalyse the acylation of sucrose in pyridine. Isopropenyl acetate was used to provide the acetyl group. The process required a large amount of enzyme to achieve a moderate yield.

Klibanov has reported that lyophilising an enzyme from a buffer solution at the optimum pH for catalytic activity, or from a solution containing the intended substrate, leads to increased activity when the enzyme is used in an anhydrous solvent.[65,66] To try this two enzymes, lipase P and subtilisin carlsberg, were both lyophilised from a phosphate buffer solution containing sucrose. They were then used to catalyse the acetylation of sucrose in pyridine and in dimethylformamide . Two different acyl donors, isopropenyl acetate and vinyl acetate, were used. The reactions were kept at 60°C and followed by t.l.c. on silica gel.

The subtilisin catalysed reactions showed a greater rate of acetylation than the lipase P catalysed reactions with the reactions carried out in dimethylformamide showing a greater degree of acylation than those in pyridine. The reactions using vinyl acetate as acyl donor proceeded faster than those using isopropenyl acetate. Perdeuterioacetylation of the reactions was

carried out as described before. Analysis showed that there was some selection in the acylations in pyridine. The lipase P catalysed reactions showed selectivity for acylation at the C-1' position whereas with subtilisin the acetylation was at the C-6 and C-6' positions. In the reactions carried out in dimethylformamide the reactions catalysed by lipase P showed selection for the C-1' position and those catalysed by subtilisin showed some selectivity towards the C-2 position.

Control experiments carried out using untreated enzyme showed that those containing the treated enzyme did have enhanced activity. Using enzyme freeze dried from phosphate buffer also produced the rate enhancement. The control experiment using just buffer salts with no enzyme showed a rate of reaction greater than that with just the reactants but not as fast as with the treated enzyme. Thus it appears that the buffer salts and the enzyme lyophilised from buffer have a catalytic effect on the reaction rate.

T.l.c. showed that the subtilisin catalysed reactions proceeded faster than those catalysed by lipase P. More products were observed probably indicating less selectivity of the position of acetylation. The apparent change in the position of acetylation with the subtilisin catalysed reactions in different solvents is also of interest.

Appendix 3: Data from the analysis of the deacetylation experiments.

Data from the deacetylation of glucose pentaacetate.

The tetraacetate class.

N.m.r. integral of each acetate signal (%) (integral of 6-acetate=100%)

Time (min)	% of total	α-anomer				β-anomer					
		4	3	6	1	2	3	2	4	6	1
40	57	97	88	100	0	85	98	97	99	100	0
60	56	100	89	100	0	88	98	87	96	100	0
75	33	100	99	100	0	98	100	92	87	100	0
90	25	100	84	100	0	84	100	91	94	100	0
100	4	100	87	100	0	89	100	97	100	100	0
150	0.5										

From the above data the composition of the tetraacetate class may be calculated.

% of tetraacetate species.

Time (min)	2,3,4,6-	1,3,4,6-	1,2,4,6-
40	54.0	4.0	3.5
60	50.0	5.5	3.0
75	30.5	2.5	0.5
90	24.5	3.0	2.0
100	3.0	0	0

The triacetate class.

N.m.r. integral of each acetate signal (%) (integral of 6-acetate=100%)

Time (min)	% of total	α-anomer				β-anomer					
		4	3	6	1	2	3	2	4	6	1
40	21	66	55	100	7	54	56	51	62	100	0
60	37	75	53	100	9	64	52	59	74	100	0
75	52	83	47	100	8	56	59	56	68	100	0
90	60	78	51	100	3	69	62	66	72	100	0
100	39	81	49	100	8	77	55	76	84	100	0
150	14	62	40		8	77	49	78	60		

The composition of the triacetate class may be calculated from the above data:

% of each triacetate species.

Time (min)	2,3,6-	2,4,6-	3,4,6-
40	7.0	6.5	4.0
60	15.5	12.0	7.0
75	21.0	18.75	8.5
90	26.0	19.0	15.0
100	20.0	11.5	9.0
150	6.5	2.0	4.5

The diacetate class.

N.m.r. integral of each acetate signal (%) (integral of 6-acetate=100%)

Time (min)	% of total	α-anomer				β-anomer					
		4	3	6	1	2	3	2	4	6	1
40	0										
60	6	27	42	100	0	30	44	24	31	100	0
75	9	22	45	100	0	32	45	28	26	100	0
90	13	29	37	100	0	33	47	27	26	100	0
100	21	19	39	100	0	42	50	33	18	100	0
150	37	15	53	100	0	32	61	29	10	100	0
150		49	32	100	0	17	36	15	49	100	0

% of each diacetate species.

Time (min)	2,6-	3,6-	4,6-
60	0	0	0
75	4.0	2.75	2.25
90	5.25	3.5	3.25
100	9.5	7.5	3.75
150	21.5	11.5	4.0

Other classes.

Time (min)	Pentaacetate	Monoacetate	Glucose
40	20	0	0
60	2.5	1.4	0.9
75	2.4	1.2	1.8
90	0.8	2.5	1.2
100	0	5.8	26.0
150	0	23.0	32.7

The deacetylation of sucrose octaacetate.

The composition of the mixture by class (%)-small scale reaction.

	Time (hours)											
Acetate class	2	4	6	8	12	24	30	38	48	60	74	102
Octaacetate	95	86	81	73	65	27	14	10	0	0	0	0
Heptaacetate	5	7	9	5	9	5	4	1	0	0	0	0
Hexaacetate	0	4	2.5	5.6	6	10.5	11	6	0	0	0	0
Pentaacetate	0	2	2.5	7.4	5	21	25	25	0	2	0	0
Tetraacetate	0	0	1.2	3.0	0	7	9	16	36	11	7	0
Triacetate	0	1	2	3	6	10	12	15	51	26	24	0
Diacetate	0	0	1	2.5	4.5	12.5	13	17	13	38	40	6
Monoacetate	0	0	0.4	0	5	6	9	8		20	23	56
Sucrose	0	0	0	0	0	0.7	3	1		3	5	38

The composition of the mixture by class (%).

	Time (hours)				
Acetate class	12	18	24	36	48
Octaacetate	60.0	43.0	27.0	0	0
Heptaacetate	9.0	6.5	5.0	2.5	0
Hexaacetate	6.0	8.5	11.6	8.8	4.1
Pentaacetate	5.0	13.5	24.9	30.7	8.7
Tetraacetate	5.0	3.0	11.3	21.9	11.2
Triacetate	6.0	7.5	8.2	15.4	14.3
Diacetate	4.5	8.5	6.8	10.6	22.8
Monoacetate	5.0	5.5	2.2	4.1	27.1
Sucrose	0	0	4.3	6.4	11.8

The heptaacetate class.

Time (hours)	N.m.r. integral of each acetate position							
	3'	2	6	6'	1'	3	4	4'
12	9.1	9.15	9.6	5	8.1	9.4	9.1	7.7
18	70	70	71	33	63	69	71	57
24	53	53	54	23	47	55	56	45
36								
48								

Analysis was carried out on these figures as described in Chapter Two. For example, taking the data for the heptaacetate class after 12 hours:

Taking the value of the C-3 acetate to be 100%, the percentage values are:

97%, 97%, 100%, 53%, 86%, 100%, 97%, 82%

respectively.The decrease in the integrals of each acetate signal corresponds to the amount of the heptaacetate species with that acetate group hydrolysed. This mixture therefore contains 47% of the 1',2,3,3',4,4',6-heptaacetate, 18% of the 1',2,3,3',4,6,6'-heptaacetate and 14% of the 2,3,3',4,4',6,6'-heptaacetate, together with small amounts of the other possible species. After 12 hours the heptaacetate class accounts for 9% of the total mixture. From this information the amount of each species in the total mixture may be calculated. For example the major heptaacetate present as 47% of the class forms 0.47x9%=4.3% of the total mixture.

% of each heptaacetate species

Time	1',2,3,3',4,4',6	2,3,3',4,4',6,6'	1',2,3,3',4,6,6'
12	4.3	1.4	1.8
18	3.45	0.7	2.4
24	3.1	1.0	1.05

The hexaacetate class.

Time (hours)	mass of mixture	N.m.r. integral of each acetate position							
		3'	2	6	6'	1'	3	4	4'
12		13.6	13.0	13.5	0.6	12.6	14.2	13.9	70.7
18		4.7	4.8	4.5	0	4.8	4.6	4.8	0
24		5.7	5.6	5.8	0.3	5.3	6.1	5.8	0.7
36		4.2	4.2	4.2	2	3.8	4.3	4.3	0
48	18mg	11.5	11.2	12	0	11.1	11.5	11.7	0
48	10mg	4.1	4.1	4.2	2.8	1.3	4.4	4.4	4

% of each hexaacetate species

time(hours)	1',2,3,3',4,6	2,3,3',4,6,6'	2,3,3',4,4',6
12	5.4		
18	8.5		
24	10.2	0.6	0.8
36	8.4	0.4	
48	3.1	1.0	

The pentaacetate class.

Time (hours)	mass of mixture	N.m.r. integral of each acetate position							
		3'	2	6	6'	1'	3	4	4'
12		9.1	8.7	8.5	0.2	0.5	9.0	9.3	0
12		4.3	1.5	4.2	0.3	3.9	4.3	3.7	0
18	21mg	9.8	9.7	9.6	0	0.4	9.6	9.6	0
18	4mg	4.7	2.8	4.3	0	2.8	4.6	3.8	0
24	40mg	7.8	8.1	8.4	0	0.5	8.0	8.0	0.5
24	3mg	4.8	1.0	4.6	0.8	5.3	5.7	5.6	0.4
36		17.8	18.0	18.2	1.0	2.2	18.2	17.5	0
48		7.8	8.4	8.7	0.1	0.3	8.9	8.7	0

% of each pentaacetate species.

Time (hours)	2,3,3',4,6	1',3,3',4,6	1',2,3,3',6	1',2,3,4,6	1',3,4,6,6'
12	3.7	1.1	0.25		
18	11.9	1.1	0.5		
24	22.9	1.5			
36	29.2	0.6	0.6		
48	8.3	0.3		0.1	0.1

The tetraacetate class.

Time (hours)	% of total mixture	N.m.r. integral of each acetate position							
		3'	2	6	6'	1'	3	4	4'
12		9.1	7.0	9.2	0	0.6	8.9	5.1	0
18	18mg	28.0	17.0	27.5	0	0	28.0	10.5	0
18	16mg	7.0	3.1	6.9	0	0	5.4	5.4	0
24	5mg	1.25	5.80	6.95	0	1.25	6.85	6.80	0.8
24	2mg	9.1	7.4	8.2	0.2	2.4	6.8	8.3	0
36		87.25	44.25	112.0	3.75	36.75	89.5	86.0	0
48		0.9	0.7	0.9	0	0	0.9	0	0

% of each tetraacetate.

Time (hours)	2,3,3',6	3,3',4,6	2,3',4,6	1',3,4,6	1',3',4,6	2,3,4,6	2,3,3',4
12	2.5	1.7	0.8				
18	1.3	1.5	0.3				
24	1.1	1.4	0.8	0.7	0.7	6.8	
36		17.5					
48	3.6						7.6

The triacetate class.

Time (hours)	% of total mixture	N.m.r. integral of each acetate position						
	3'	2	6	6'	1'	3	4	4'
12								
18	143	131	36	2	9	129	28	0
24								
36	83	37	76	4	40	46	32	0
36	87	62	151	8	47	86	81	0
48	16	12	16	0	0	3	0.5	0

% of each triacetate species.

Time (hours)	2,3,3'	3,3',6	3',4,6	2,3',6
18	4.6	(	3.4	)
24				
36		**	**	
48	10.9			3.4

The triacetate sample for 24 hours was contaminated and so data could not be calculated. The class for 36 hours cannot be solved from the data, although the species indicated by ** are present.
The diacetate, monoacetate and sucrose composition is shown by the data at the beginning of the section.

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